Molecular and Biochemical Studies on Self-incompatibility of Two Commercially Important Cherry Species (*Prunus avium* L. and *P. cerasus* L.)

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Contents

General Introduction

There are two major cultivated cherry species, diploid sweet cherry (*Prunus avium* L.) and tetraploid sour cherry (*P. cerasus* L.). Although cherry production fluctuates between years, maximum world production values for sweet and sour cherries are approximately 1,450,000 and 1,600,000 tons, respectively. In Japan, sweet cherry is one of important fruit crop and its production was 21,200 tons covering a production area of about 3,970 ha in 2001 (Source: Ministry of Agriculture, Forestry and Fisheries, Minister's Secretariat, Statistics and Information Department). The production area in Japan is increasing consistently in the last ten years (Source: Zennoh Yamagata).

Sweet and sour cherries are reported to have originated in the Near East center, which includes Iran, Iraq, and Syria (Vavilov, 1951). Watkins (1976) suggests that the first diploid *Prunus* species arose in central Asia, and that sweet and sour cherries were early derivatives of this ancestral *Prunus*. Ground cherry (*P. fruticosa*) and sweet cherry are believed to be the progenitor species of sour cherry (Olden and Nybom, 1968). As sweet and sour cherries spread throughout Europe, ecotypes arose within each species that differed in cold hardiness, tree habit, and fruit and leaf characteristics (Kolesnikova, 1975). In Japan, some cherry cultivars were introduced from Europe and USA in the early Meiji era, and have been grown in apple growing regions such as Yamagata. After World War II, sweet cherry improvement started in Japan by breeding program or selection of chance seedlings. In 1990, 17 cultivars were selected for commercial production and now the number of cultivars is increasing with some new cultivars mostly derived from seedlings of the breeding program at Yamagata Prefectural Horticultural Experimental Station. Although the sour cherry market is bigger than the sweet cherry market in the world, sour cherry is rarely grown commercially in Japan because sour cherry suitable to processed use does not meet Japan domestic demand for fresh fruit. Most sour cherries are processed to pie or yogurt, whereas sweet cherries are consumed mostly fresh.

Sweet cherry cultivars are predominantly self-incompatible with several self-compatible lines artificially produced through irradiation breeding. Self-incompatibility in sweet cherry was first identified by East and Mangelsdorf

(1925) and later determined to be of the gametophytic type with the multiple allelic *S*-locus (Lewis, 1948). In contrast to sweet cherry, sour cherry cultivars are predominantly self-compatible with several self-incompatible cultivars. Self-incompatibility in sour cherry is characterized by inhibition of pollen tube growth in style, which is typical phenomenon of gametophytic self-incompatibility (Lansari and Iezzoni, 1989).

Cherry production is hampered by self-incompatibility because cherries are unable to bear fruit parthenocarpically and fertilization is indispensable for their fruit production. To ensure fruit set, pollinizer trees are inter-planted in the commercial orchard and beehives are introduced. In Japan, alternatively, hand-pollination is practiced although it is a very labor-intensive operation. Self-incompatibility is also a hindrance to breeders wishing to carry out selfing or crossing between cultivars in the same pollination incompatibility groups. One of the breeding objectives of cherries, therefore, is the production of self-compatible cultivars. So far, several self-compatible sweet cherry cultivars, such as 'Stella' and its offspring, have been produced (Lapins, 1970, 1975). Although sour cherry is predominantly self-compatible, self-incompatible selections can result from crosses between two self-compatible parents. Since any successful new sour cherry cultivars would have to be self-compatible, self-incompatibility is also a hindrance to sour cherry breeder.

In gametophytic self-incompatibility, the pistil distinguishes between self and non-self pollen based on whether the *S*-haplotype of the haploid pollen matches either of the two *S*-haplotypes of the diploid pistil (Figs. 1, 2). Although the gametophytic self-incompatibility had been believed to be controlled by a single genetic locus (*S*-locus), recent studies revealed that *S*-locus consists of at least two different genes for the pistil and pollen components that are responsible for self-incompatible reaction (for a review, see McCubbin and Kao, 2000). They are tightly linked to each other as if they were "a single gene". Thus the term haplotype is used to denote variants of the locus, and the term allele is used to denote variants of a given polymorphic gene at the *S*-locus. When haplotypes match, pollen is recognized by the pistil as self and rejected, whereas if haplotypes differ, pollen is accepted for fertilization. Thus, crosses between two plants are compatible as long as their *S*-haplotypes differ in one of the two *S*-haplotypes (Figs. 1, 2).

The molecular mechanism of gametophytic self-incompatibility has been studied extensively in solanaceous plant species. cDNAs encoding the pistil proteins that were associated with *S*-haplotype were first cloned from *Nicotiana alata* (Anderson et al., 1986). Because the deduced amino acid sequences contained an active site of the fungal RNases T2 (Kawata et al., 1988) and Rh (Horiuchi et al., 1988), it was suggested that stylar RNases were involved in the gametophytic self-incompatible reaction (McClure et al., 1989). Since this finding, the *S*-gene products in pistils of Solanaceae are called S-RNases. The results from transgenic analyses clearly demonstrated that S-RNase is a pistil determinant for the pollen rejection response in Solanaceae (McCubbin et al., 1997; Lee et al., 1994; Murfett et al., 1994, 1995).

Recently, it has been shown that S-RNases are also associated with the gametophytic self-incompatibility of Japanese pear (*Pyrus pyrifolia*), apple (*Malus* × *domestica*) and European pear (*Pyrus communis*), which belong to the subfamily Maloideae of the family Rosaceae as is the case with Solanaceae (for a review, see McCubbin and Kao, 2000). Because *Prunus*, to which sweet cherry belongs, is one of the Rosaceae genera, S-RNase may play a role in rejecting self pollen tube growth as in apple and pear.

Identification and characterization of S-RNases in cherries would help not only to elucidate the physiological and molecular mechanisms of gametophytic self-incompatibility in cherries but also to develop molecular typing methods for *S*-haplotypes in cherries. RFLP and PCR analyses for S-RNase genes have been successfully used to type *S*-haplotypes of apple and Japanese pear (Janssens et al., 1995; Matsumoto et al., 1999; Sakurai et al., 1997; Ishimizu et al., 1999). Furthermore, molecular breeding of self-compatible cherries through gene transfer techniques would become possible if cDNAs for S-RNases of sweet cherry are cloned. In solanaceous plant species, antisense expression of cDNAs for S-RNases results in self-compatible phenotype (Lee et al., 1994; Murfett et al., 1995).

This study was designed to identify and characterize S-RNase of sweet cherry, which is a pistil determinant of gametophytic self-incompatibility (Chapter 1), and to develop molecular typing methods for its *S*-haplotypes from the information obtained (Chapter 2). S-RNases of sour cherry were also identified to clarify genetic control of

self-incompatibility and self-compatibility in tetraploid sour cherry (Chapter 3). Finally, evolutional background of self-incompatibility and S-RNase molecules in *Prunus* species were discussed based on the information obtained from stylar non-S-RNases of sweet cherry (Chapter 4).

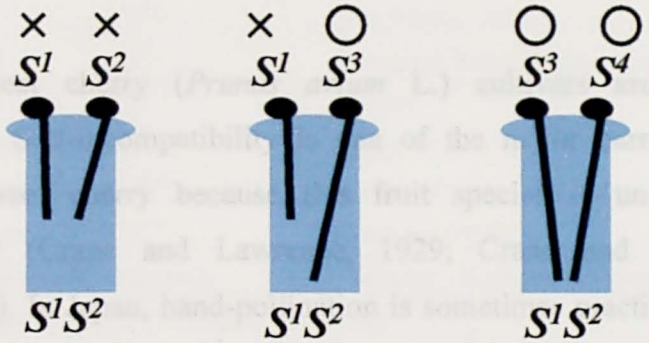

Fig. 1. Genetic control of gametophytic self-incompatibility. The pollen SI phenotype is determined by the *S*-genotype of the pollen. The S^1S^2, S^1S^3, and S^3S^4 plant generates the pollen whose SI phenotypes are S^1 *or* S^2, S^1 *or* S^3, and S^3 *or* S^4, respectively. The S^1- and S^2-pollen tube growth is rejected in the style of S^1S^2 plant, but S^3- and S^4-pollen tubes can grow in the style of S^1S^2 plant.

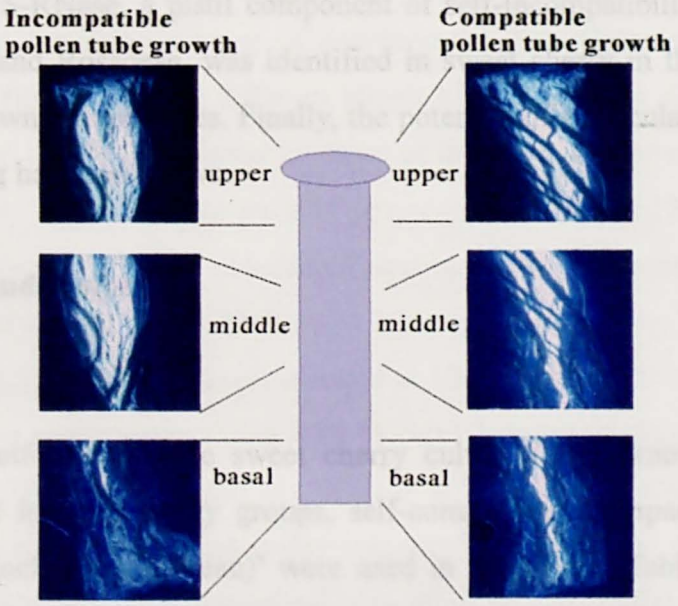

Fig. 2. Pollen tube growth in sweet cherry (*P. avium*) pistil. Pistils (Satonishiki, S^3S^6) that were pollinated with compatible (Napoleon, S^3S^4) and incompatible pollen (Satonishiki, S^3S^6) grains were collected 72 h after pollination and immersed in fixing solution for 24 h, incubated in 10 N NaOH for 5 h, then stained in 0.1 % aniline blue. Pollen tubes were observed by ultraviolet fluorescent microscopy. The upper, middle, and basal segment of the pistils were photographed. The part of style where observations were made is illustrated in the middle figure. Note that there are no pollen tubes in basal part of the pistil from incompatible cross, but that strong fluorescences of pollen tubes in basal part were observed from compatible cross.

Chapter 1
Identification and characterization of S-RNases associated with gametophytic self-incompatibility in sweet cherry (*Prunus avium* L.)

1.1. Introduction

Most sweet cherry (*Prunus avium* L.) cultivars are gametophytically self-incompatible. Self-incompatibility is one of the major barriers to stable fruit production of sweet cherry because this fruit species is unable to bear fruit parthenocarpically (Crane and Lawrence, 1929; Crane and Brown, 1937; de Nettancourt, 1977). In Japan, hand-pollination is sometimes practiced although it is a very labor-intensive operation. Alternatively, cross-compatible cultivars that flower simultaneously are inter-planted in commercial orchards to ensure cross-pollination. To cope with self-incompatibility in sweet cherry, we started to elucidate the molecular mechanism of the gametophytic self-incompatibility in sweet cherry. As a first step towards this end, S-RNase, a pistil component of self-incompatibility in Solanaceae, Scrophulariaceae and Rosaceae, was identified in sweet cherry in this chapter, using cultivars with known *S*-haplotypes. Finally, the potential of molecular analyses for the *S*-haplotype typing has been shown.

1.2. Materials and Methods

Plant material

Fifteen self-incompatible sweet cherry cultivars with known *S*-haplotypes, representing eight incompatibility groups, self-compatible 'Compact Stella' (S^3S^{4m}), and 'Takasago (Rockport Bigarreau)' were used in this study (Table 1.1). 'Compact Stella' (Lapins, 1975) originated as a mutant with semidwarf growth habit, through X-ray irradiation of dormant scions of self-compatible 'Stella' (S^3S^{4m}) cherry (Lapins, 1970). As does 'Stella', 'Compact Stella' loses the function of pollen S^4-allele, but not that of pistil S^4-allele. Thus 'Compact Stella' is a universal pollinator but rejects both S^3 and S^4-pollen tube growth in its style (Lapins, 1975). All the cultivars tested were grown at Yamagata Prefectural Horticultural Experimental Station.

Table 1.1. Sweet cherry cultivars used and their *S*-haplotypes

IG[z]	*S*-haplotype	Cultivar
I	S^1S^2	Early Rivers
II	S^1S^3	Gilpeck, Van
III	S^3S^4	Bing, Napoleon
IV	S^2S^3	Sue, Victor, Velvet
VI	S^3S^6	Governor Wood, Nanyo, Satonishiki
IX	S^1S^4	Rainier
XIII	S^2S^4	Peggy Rivers
XVI	$S^3S^{9\,y}$	Burlat, Moreau
none	S^3S^{4m}	Compact Stella
unknown	unknown	Takasago (Rockport Bigarreau)

[z]Incompatibility group (Iezzoni et al., 2002)

[y]Currently used worldwide standard nomenclature of *S*-haplotypes that is different from the originally reported ones (see Appendix 1)

Protein assay

Styles with stigmas of all the cultivars listed in Table 1.1 were dissected from the flower buds at the balloon stage of development. For 'Satonishiki', styles with stigmas from the flower buds at different stages of development were also used. In addition, the upper, middle, and lower portions of the styles dissected from the flower buds of 'Satonishiki' at the balloon stage of development were used for the experiment. These samples were crushed in pre-chilled acetone (-20 °C) containing 8 % (w/v) trichloroacetic acid (TCA) plus 0.07 % (v/v) 2-mercaptoethanol, and incubated for 30 min at -20 °C. The suspension was centrifuged at 10000 × g for 10 min, and the pellet was resuspended in acetone containing 0.07 % (v/v) 2-mercaptoethanol and incubated 1 h at -20 °C. After centrifugation at 10000 × g for 10 min, the supernatant was discarded and the pellet was dried under vacuum, then used for protein extraction. Acetone powder (1 mg) was homogenized with 80 µl of lysis buffer (O'Farrell, 1975) consisting of 9.5 M urea, 2 % (v/v) Nonidet P-40 (Nacalai tesque, Kyoto), 2 % (v/v) Ampholine pH 3.5-10 (Amersham Biosciences, Tokyo), 5 % (v/v) 2-mercaptoethanol, and 5 % (w/v) polyvinylpyrrolidone K30 (Nacalai tesque, Kyoto). After incubation at 60 °C for 10 min, the supernatant was isolated by centrifugation at 10000 × g for 10 min, and 50 µl aliquots was subjected to two dimensional polyacrylamide gel

electrophoresis (2D-PAGE) using nonequilibrium pH gradient electrophoresis (NEPHGE) for the first dimension and SDS-PAGE for the second dimension as described by O'Farrell et al (1977) with slight modifications. The samples were applied to the acidic end of the first dimensional gels consisting of 4 % (w/v) acrylamide, 0.2 % (w/v) bis-acrylamide, 8 M urea, 2 % (v/v) Nonidet P-40, 2 % (v/v) Ampholine pH 3.5-10, and 4 % (v/v) Pharmalyte pH 8-10.5 (Amersham Biosciences, Tokyo). NEPHGE was conducted at 200 V for 20 min, at 300 V for 20 min, at 400 V for 2 h, and finally at 800 V for 30 min. After electrophoresis, the gels were equilibrated with the SDS-sample buffer and applied to 15 % SDS-PAGE for the second dimensional electrophoresis. Proteins in the gel were detected by silver staining using Sil-Best Stain for Protein/PAGE (Nacalai tesque, Kyoto). For 'Satonishiki', proteins separated by 2D-PAGE were also electroblotted onto a PVDF membrane (Hirano and Watanabe, 1990) and glycoproteins were detected with biotin-conjugated concanavalin A and horseradish peroxidase-conjugated streptavidin. Peroxidase acitivity on the PVDF membrane was visualized using 4-chloro-1-naphthol as a substrate. Immunodetection of proteins electroblotted onto PVDF membrane was also conducted for 'Satonishiki' and 'Velvet' by using mouse anti-S^4-serum prepared against S^4-RNase of Japanese pear (Sassa et al., 1993). In addition, for these cultivars, portions of the PVDF membrane carrying the proteins of interest were also cut out after Coomassie Blue staining and subjected to a gas-phase protein sequencer (476A, Applied Biosystems, Tokyo) for N-terminal amino acid sequence analysis. Edman degradation was performed according to the standard program obtained from Applied Biosystems.

cDNA cloning for S-RNases

Total RNA was isolated from styles with stigmas of 'Rainier' and 'Satonishiki' at the balloon stage of development as described by McClure et al. (1990) with several minor modifications. Poly $(A)^+$ RNA was isolated from the total RNA using Oligotex-dT30 (TaKaRa Shuzo Co., Shiga). Double-stranded cDNA was synthesized from the poly $(A)^+$ RNA, cloned into Lambda ZAPII vector (Stratagene, LaJolla, Calif.), and packaged in vitro using MaxPlax Packaging Extract Kit (Epicentre Technologies, Madison, Wis.).

A partially degenerate primer (AS1: 5'-TATTT TCAAT TTGTN CAGCA ATGG-3') was designed based on the N-terminal amino acid sequences of putative *S*-proteins of sweet cherry, considering the corresponding cDNA sequences of S-RNases of Japanese pear (Sassa and Hirano, 1997) and apple (Sassa et al., 1996). The primer was used in 3' rapid amplification of cDNA ends (3'RACE) with a commercial kit (3'RACE System for Rapid Amplification of cDNA Ends, Invitrogen, Tokyo). One microgram of total RNA from the styles with stigmas of 'Satonishiki' was used for first strand cDNA synthesis. PCR was performed using a program of 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min 30 s with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 5 min. The reaction mixture for PCR contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 200 nM each of primers, the template cDNA equivalent to amount synthesized from 0.1 μg of the total RNA, and 1 U of TaKaRa Ex Taq polymerase (TaKaRa Shuzo Co., Shiga) in a 50 μl reaction volume. The PCR products were subcloned into the TA cloning vector (pGEM-T Easy vector system; Promega, Madison, Wis.). Nucleotide sequences of the inserts of several clones were determined using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) and it was confirmed that they include DNA sequence encoding N-terminal amino acid sequence of putative *S*-proteins of sweet cherry. The insert was further PCR amplified with a primer set, Pru-C2 (5'- CTATG GCCAA GTAAT TATTC AAACC -3') and Pru-C5 (5'- TACCA CTTCA TGTAA CAACT GAG -3'), which were designed from the DNA sequence corresponding to the previously identified C2 and C5 conserved domains of solanaceous S-RNases (Tsai et al., 1992). PCR was performed using a program of 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The PCR reaction mixture was the same as described above except that the concentration of primers was increased to 400 nM and 10 ng of plasmid was used as a template. The amplified fragment of approximately 400 bp was labeled by random primer incorporation of DIG-dUTP (Roche, Tokyo) and used as a probe to screen the primary cDNA library from 'Satonishiki' and 'Rainier' without amplification, which consisted of 1×10^5 and 3×10^6 plaque forming unit (pfu)/ml, respectively. Petri dishes (90 mm

in diameter), each contained approximately 1000 plaques, were screened by plaque hybridization. After the low stringency washes (2 × 5 min at room temperature with 5 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 °C with 1 × SSC and 0.1 % SDS), colorimetric detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was conducted according to the manufacturer's protocol (Roche, Tokyo). Selected clones were converted to pBluescript plasmids according to the manufacturer's protocol (Stratagene, Tokyo) and DNA sequences were determined as described above.

One microgram of total RNA was used for first strand cDNA synthesis by SuperScript II RT (Invitrogen, Tokyo) with a primer Adp-dT (5'- CGACG TTGTA AAACG ACGGC CAGTT TTTTT TTTTT TTTT -3'), which comprises M13-20 sequence primer (5'- CGACG TTGTA AAACG ACGGC CAGT -3') and oligo $(dT)_{15}$. A primer, Pru-T2 (5'- TSTTS TTGST TTTGC TTTCT TC -3'), derived from the DNA sequence corresponding to the signal peptide sequence of S-RNases of sweet cherry was used in 3'RACE with M13-20 primer as an adapter primer. PCR was performed using a program of 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The reaction mixture for PCR was described above. The PCR products were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, Wis.). Nucleotide sequences of the inserts of several clones were determined as described above.

Genomic DNA blot analysis

Total DNA was isolated from young leaves by the CTAB method (Doyle and Doyle, 1987), using all the cultivars listed in Table 1.1. After purifying the isolated DNA by PEG precipitation (Mak and Ho, 1993), 5 μg of DNA was digested either with *Eco*RI or *Hin*dIII and run on 0.8 % agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Biosciences, Tokyo, Japan), and probed with the two different regions of the cDNAs, the C2-C5 and C5f-polyA fragments. The C2-C5 fragments of cDNAs were PCR labeled by DIG-dUTP with the Pru-C2 and Pru-C5 primers. The C5f-polyA fragments of 3'RACE clones were also labeled by PCR with the Pru-C5f (5'- GTTGTTACATGAAGTGGTATTTTGTTATG -3') and M13-20 primers. After

high stringency washes (2 × 5 min at room temperature with 2 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 °C with 0.1 ×SSC and 0.1 % SDS), immunological detection of the hybridization was carried out using the anti-DIG-alkaline phosphate conjugate and the chemiluminescent substrate CSPD (Roche, Tokyo). Chemiluminescence was documented on X-ray films.

RNA blot analysis

Styles with stigmas, ovaries, petals, calyxes, peduncles, and anthers were dissected from the flower buds of 'Satonishiki' at the balloon stage of development. Young leaves of 'Satonishiki' were collected in spring. Total RNA was isolated from these samples as described above. Twenty micrograms of the total RNA was run in a formaldehyde (1 %) agarose gel, blotted onto a nylon membrane, and probed with two different regions of the cDNAs, C2-C5 and C5f-polyA fragments of the cDNAs. After high stringency washes (2 × 5 min at room temperature with 2 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 °C with 0.1 ×SSC and 0.1 % SDS), immunological detection of the hybridization was carried out as described above for genomic DNA blot analysis.

Amplification of S-alleles by PCR

As described above for the genomic DNA blot analysis, total DNA was isolated and purified from all the cultivars listed in Table 1.1 and used as template DNA for PCR. PCR was performed using a program of 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min 30 s with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The reaction mixture for PCR contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 400 nM each of primers, 50 ng of template cDNA, and 1 U of TaKaRa Ex Taq polymerase (TaKaRa Shuzo Co., Shiga, Japan) in a 50 μl reaction volume. Two different primer sets, Pru-T2 and Pru-C4R (5'- GGATGTGGTACGATTGAAGCG -3'), and Pru-C2 and Pru-C4R, were used. After PCR, the PCR products were run on 1.5 % agarose gel and DNA bands were visualized by ethidium bromide staining. Then the DNA in the gel was blotted onto a nylon membrane and probed with the C2-C5 fragments of S^2-RNase cDNA to confirm that the bands observed were truly from pistil *S*-genes. The

C2-C5 fragments of S^2-RNase cDNA were labeled by DIG-dUTP as described above. After high stringency washes (2 × 5 min at room temperature with 5 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 °C with 1 × SSC and 0.1 % SDS), colorimetric detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was conducted according to the manufacturer's protocol (Roche, Tokyo).

1.3. Results and Discussion

Protein assay

A group of proteins with M_r and pI similar to those of S-RNase of almond (Tao et al., 1997) were found in the 2D-PAGE profiles after silver staining (Fig. 1.1). These proteins were present at highest concentration in the upper segments of the styles (Fig. 1.2A). Furthermore, concentrations of these proteins became higher as styles matured (Fig. 1.2B), which correlates with the ability of the style to reject self-incompatible pollen tube growth. It has been reported that incompatible pollen tube growth cannot be fully inhibited by young immature styles of Japanese pear (Hiratsuka and Hirata, 1985). It was found that these proteins contained sugar chains that react with concanavalin A (Fig. 1.3A). In this putative *S*-glycoprotein group, there were at least four different glycoproteins (the spots I, II, III, and IV) with different M_r and pI values (Fig. 1.3B). Two or three of them were seen in each cultivar, and cultivars with the same *S*-haplotype yielded the same spots. 'Compact Stella' showed the same spot pattern as S^3S^4 cultivars (Fig. 1.1), which is consistent with the observation that both pistil S^3- and S^{4m}-alleles function in 'Compact Stella'. Among the *S*-glycoprotein group, spots associated with S^2- and S^6-haplotypes could be found. The most basic *S*-protein spot IV was only observed in the cultivars with S^2-haplotype. The second basic spot III was observed only in the cultivars with S^6-haplotype (Fig. 1.1, 1.3B). Thus the former seemed to be a pistil S^2-allelic product and the latter to be a pistil S^6 product. For 'Satonishiki' and 'Velvet', the putative pistil S^2- and S^6-allelic products along with two other *S*-glycoprotein spots (the spots A and B of 'Velvet' and the spots A' and B' of 'Satonishiki') reacted with the anti-S^4-RNase serum, and were shown to be immunologically similar to S-RNase of Japanese pear (Fig. 1.3C). There

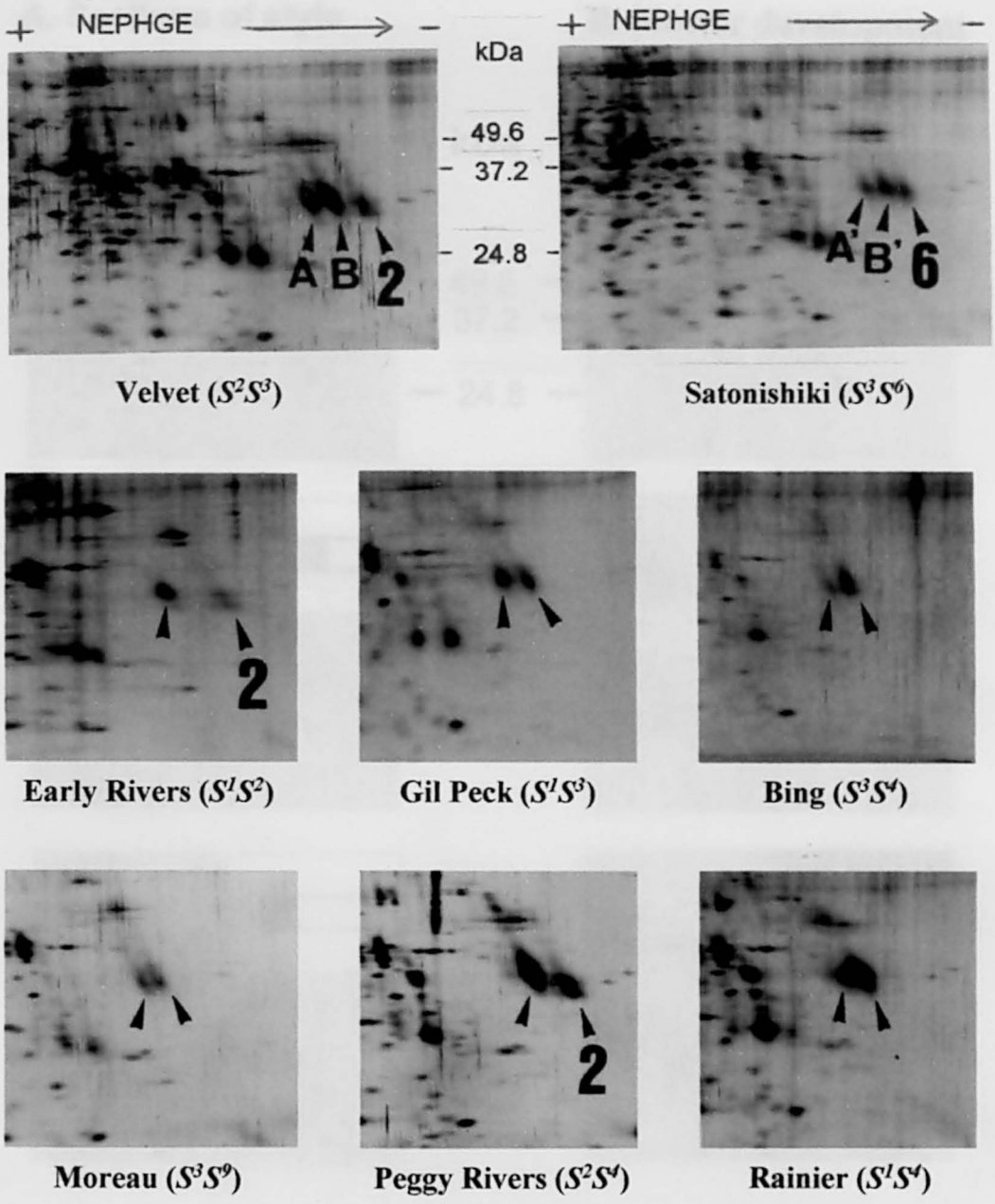

Fig. 1.1. Identification and characterization of *S*-glycoproteins of sweet cherry cultivars. Stylar proteins were separated by 2D-PAGE and detected by silver staining. Spots A, A', B, and B' were assigned to be the S^3-protein (see text).

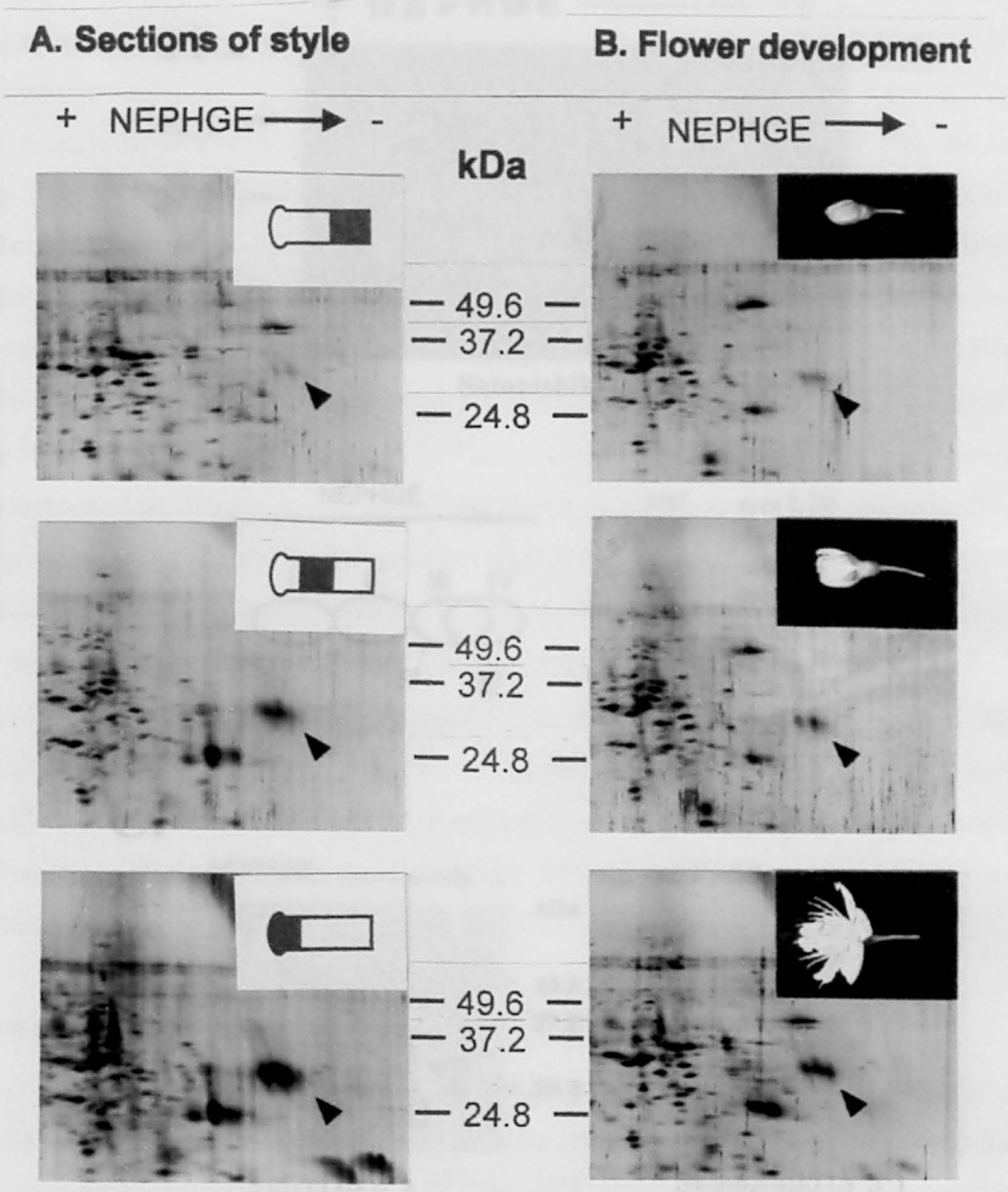

Fig. 1.2. Spatial (A) and temporal (B) regulations of *S*-glycoprotein expression in 'Satonishiki' sweet cherry (S^3S^6). Crude extracts prepared from the lower, middle, and upper portions of the styles (A) or styles with stigmas at different stages (white bud, balloon, and full bloom) of flower bud development (B) were subjected to 2D-PAGE and proteins were detected by silver staining. *S*-glycoproteins are marked with *arrowheads*.

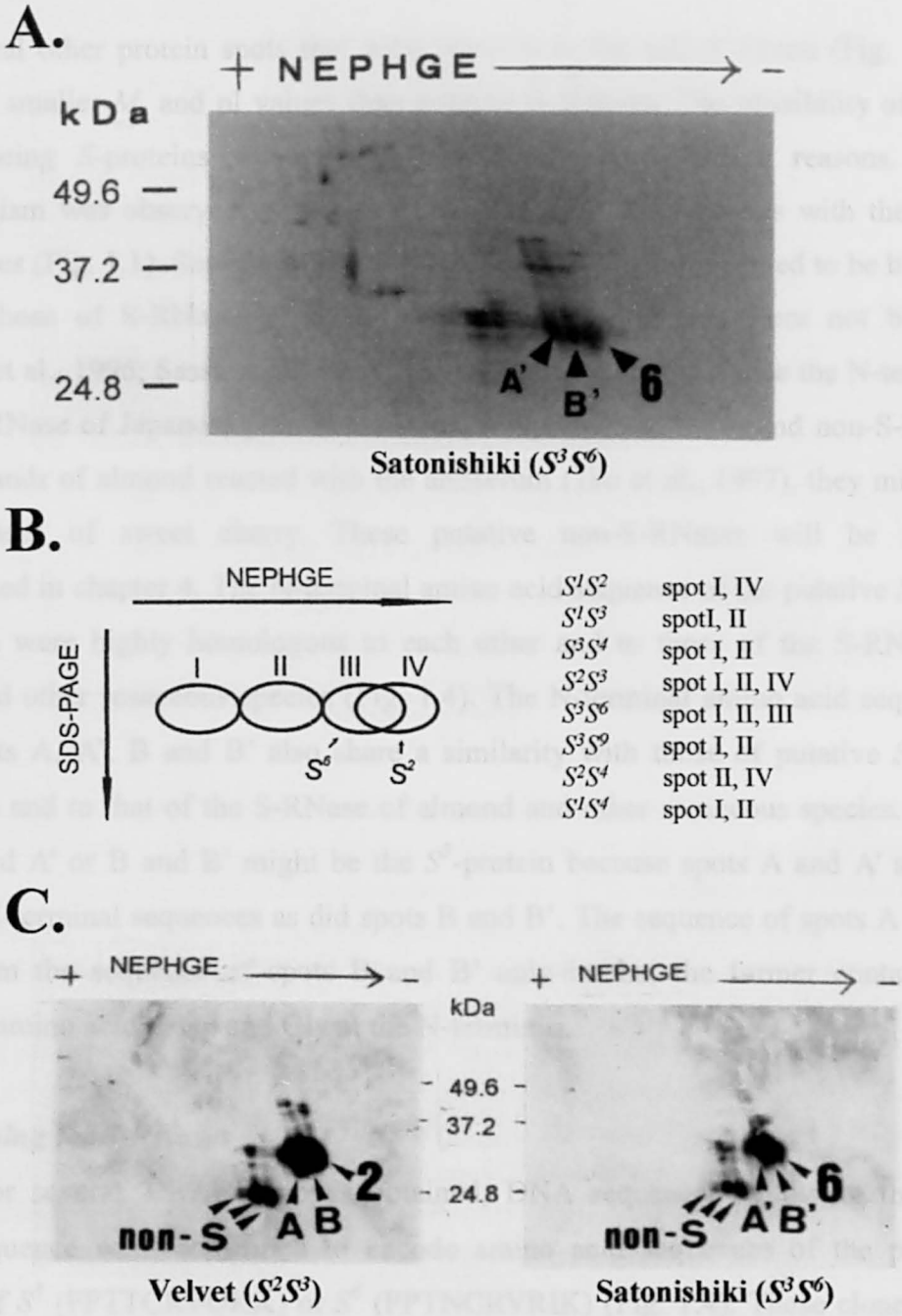

Fig. 1.3. Stylar proteins were separated by 2D-PAGE. Proteins in the 2D-PAGE gel from 'Satonishiki' were blotted to a PVDF membrane and detected by glycoprotein staining with concanavalin A (A). Proteins in the 2D-PAGE gel from 'Satonishiki' and 'Velvet' were blotted to a PVDF membrane and immunodetected with the anti-S^4-serum prepared from Japanese pear S^4-RNase (C). Schematic diagram (B) is of the distribution of sweet cherry *S*-glycoproteins in 2D-PAGE profiles.

were several other protein spots that were reactive to the anti-S^4-serum (Fig. 1.3C), which had smaller M_r and pI values than putative S-RNases. The possibility of these proteins being *S*-proteins was ruled out for the following two reasons. First, polymorphism was observed with these proteins among the cultivars with the same *S*-haplotypes (Fig. 1.1). Second, the N-terminus of these proteins seemed to be blocked although those of S-RNases reported so far for the Rosaceae were not blocked (Ishimizu et al., 1996; Sassa et al., 1993; Tomimoto et al., 1996). Since the N-terminal of non-S-RNase of Japanese pear is blocked (Norioka et al., 1996) and non-S-RNase acitivity bands of almond reacted with the antiserum (Tao et al., 1997), they might be non-S-RNases of sweet cherry. These putative non-S-RNases will be further characterized in chapter 4. The N-terminal amino acid sequence of the putative S^2- and S^6-proteins were highly homologous to each other and to those of the S-RNase of almond and other rosaceous species (Fig. 1.4). The N-terminal amino acid sequences of the spots A, A', B and B' also share a similarity with those of putative S^2- and S^6-proteins and to that of the S-RNase of almond and other rosaceous species. Either spots A and A' or B and B' might be the S^3-protein because spots A and A' showed identical N-terminal sequences as did spots B and B'. The sequence of spots A and A' differs from the sequence of spots B and B' only in that the former contain two additional amino acids, Asp and Gly at the N-terminus.

cDNA cloning for S-RNases

For several 3'RACE clones obtained, DNA sequences following the AS1 primer sequence were confirmed to encode amino acid sequences of the putative products of S^3 (PPTTCRVQKK) or S^6 (PPTNCRVRIK) (Fig. 1.4). These clones were further PCR amplified by the Pru-C2 and Pru-C5 primers (Fig. 1.5) and used as probes to screen the cDNA library from 'Satonishiki'. About 90000 plaques were screened by these probes and seven positive plaques with an insert length longer than 700 bp were obtained. Under the low stringency wash condition we used, these plaques hybridized to both probes. When these were converted to pBluescript and PCR amplified by the Pru-C2 and Pru-C5 primers, all seven clones gave PCR products of approximately 400 bp, the expected size from the 3'RACE clones used for probes. *Mbo*I digests of the PCR products showed that these were identical to 3'RACE clones; six of them gave

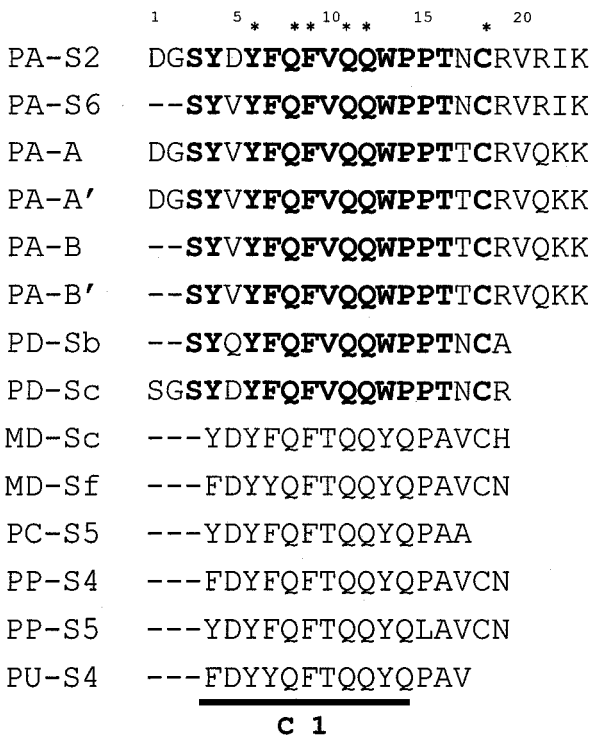

Fig. 1.4. Alignment of N-terminal amino acid sequences of S-proteins of sweet cherry and other rosaceous *S*-RNases. Amino acid sequences corresponding to the C1, previously reported conserved domain of *S*-RNases in Solanaceae (Ioerger et al., 1991) and Rosaceae (Norioka et al., 1996; Ushijima et al., 1998), are indicated at the bottom. Amino acid residues conserved in all *Prunus S*-RNases are indicated by bold face and those conserved in all rosaceous *S*-RNases are marked with *asterisks*. Plant species from which each sequence is derived are denoted by the initials of their scientific names, i.e., PA refers to *Prunus avium*. Sequence data for the S-RNases included are as follows: PD-Sb and Sc [S^b and S^c–RNases of *Prunus dulcis* (almond) (Ushijima et al., 1998)]; MD-Sc and Sf [S^c and S^f–RNases of *Malus x domestica* (apple) (Sassa et al., 1996); PC-S5 [S^5–RNase of *Pyrus communis* (European pear) (Tomimoto et al., 1996)]; PP-S4 and S5 [S^4 and S^5–RNases of *Pyrus pyrifolia* (Japanese pear) (Sassa et al., 1996; Sassa and Hirano, 1997)]; PU-S4 [S^4–RNase of *Pyrus ussuriensis* (Chinese pear) (Tomimoto et al., 1996)].

the same restriction fragment pattern as that of the 3'RACE clone encoding the putative pistil S^3-allelic product and one gave the same restriction fragment pattern as that of the 3'RACE clone encoding the putative pistil S^6-allelic product (data not shown). DNA sequencing of two clones for the former and one for the latter confirmed that they contained the sequences encoding the N-terminal amino acid sequences of the putative pistil S^3- and S^6-allelic products, respectively. Although the 3' untranslated

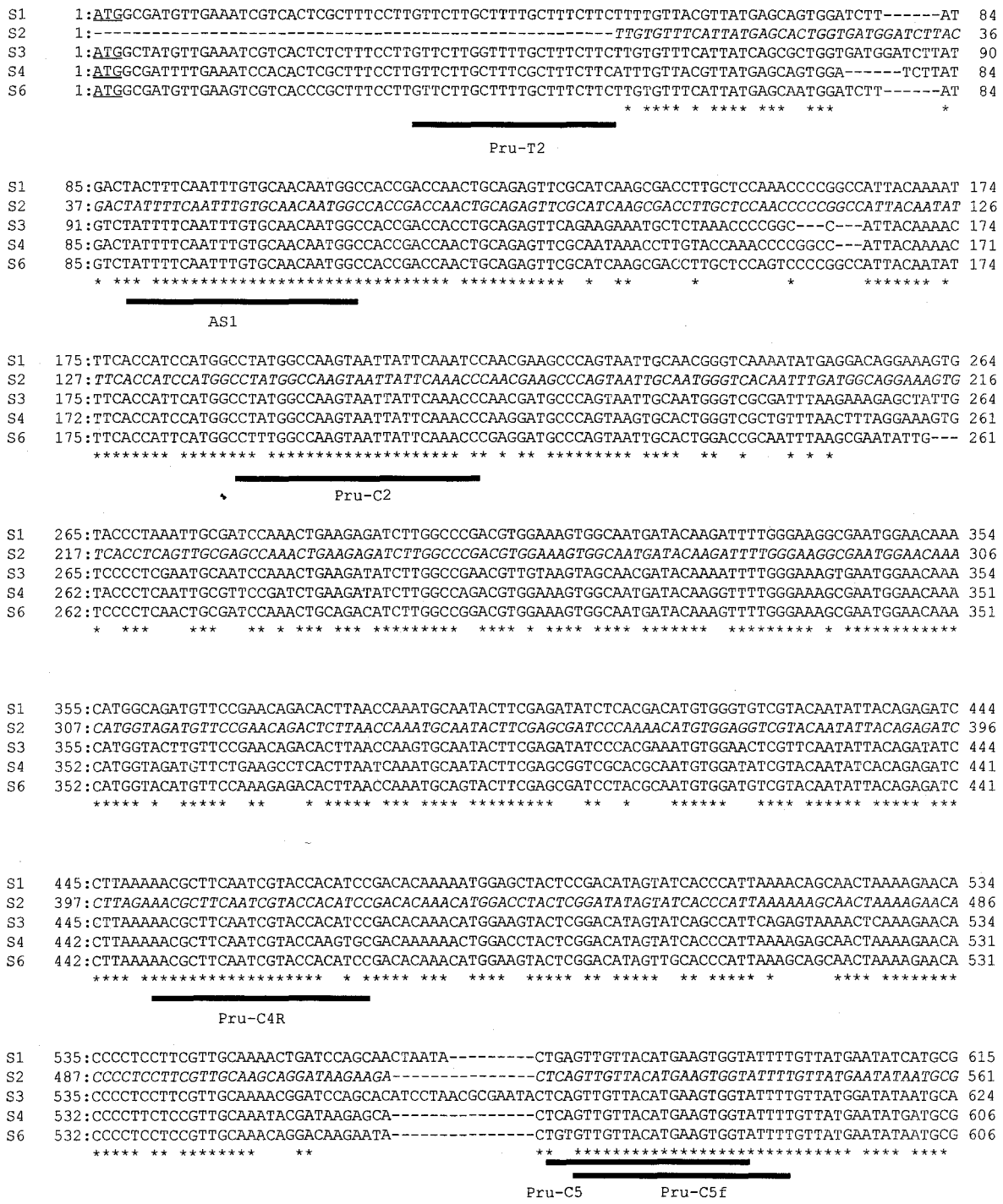

Fig. 1.5. (continues to next page)

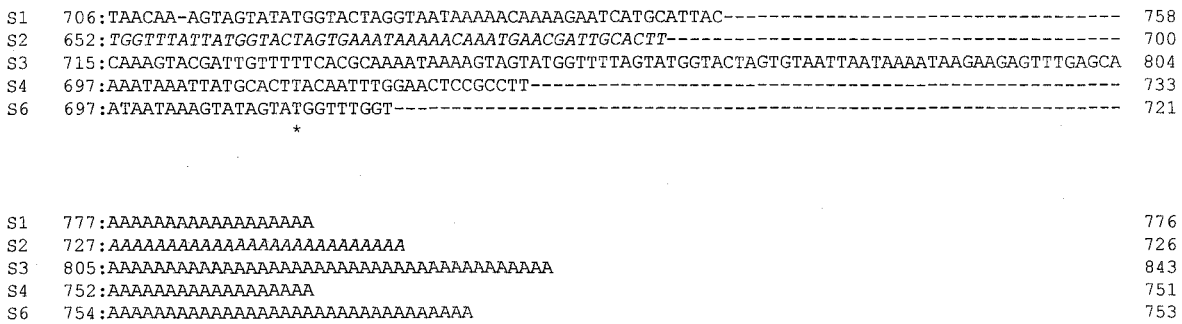

Fig. 1.5. Nucleotide sequences of the S^1-, S^2-, S^3-, S^4- and S^6-RNase cDNAs of sweet cherry. The alignment was generated by CLUSTAL X version 1.6 (Thompson et al., 1997). Gaps are marked by *dashes*. Conserved nucleotides are indicated by asterisks. Nucleotide sequences shown in *italic* are only from the 3'RACE clones and those shown in gothic are from the clones screened from the cDNA library. The initiation and stop codons are underlined. Sequences used to design oligonucleotide primers are indicated with the name of primers (Pru-T2, AS1, Pru-C2, Pru-C4R, Pru-C5, and Pru-C5f).

region of the putative S^6-cDNA clone was truncated, both the cDNA clones contained the entire coding sequences (Fig. 1.6). The putative pistil S^3- and S^6-cDNAs include open reading frames of 687 and 669 nucleotides (Fig. 1.5), which encode 229 and 223 amino acid residues (Fig. 1.6), respectively. Deduced amino acid sequences from the cDNAs contained putative signal peptide comprising of approximately 25 amino acid residues at the N-terminal (Fig. 1.6).

The Pru-T2 primer was designed from the DNA sequences corresponding to the signal peptide sequence of the putative pistil S^3- and S^6-cDNAs of 'Satonishiki' (Fig. 1.5) and used for 3'RACE to obtain information on the 3' untranslated regions and to compare the *S*-alleles of different cultivars. The Pru-T2 primer was successfully used to obtain 3'RACE clones encoding putative pistil S^2- and S^3-cDNAs of 'Velvet' and S^3- and S^6-cDNAs of 'Satonishiki'. For 'Velvet', 24 independent 3'RACE clones of approximately 750 bp that could be PCR amplified with the Pru-C2 and Pru-C5 primers were obtained. *Mbo*I digest of the amplified products suggested that one of them was identical to the putative pistil S^3-cDNA from the cDNA library of 'Satonishiki'. DNA sequencing confirmed that they contained exactly the same sequence as the putative pistil S^3-cDNA from the cDNA library of 'Satonishiki' (Fig. 1.5). The remaining 23 clones yielded the same restriction fragments but the fragment

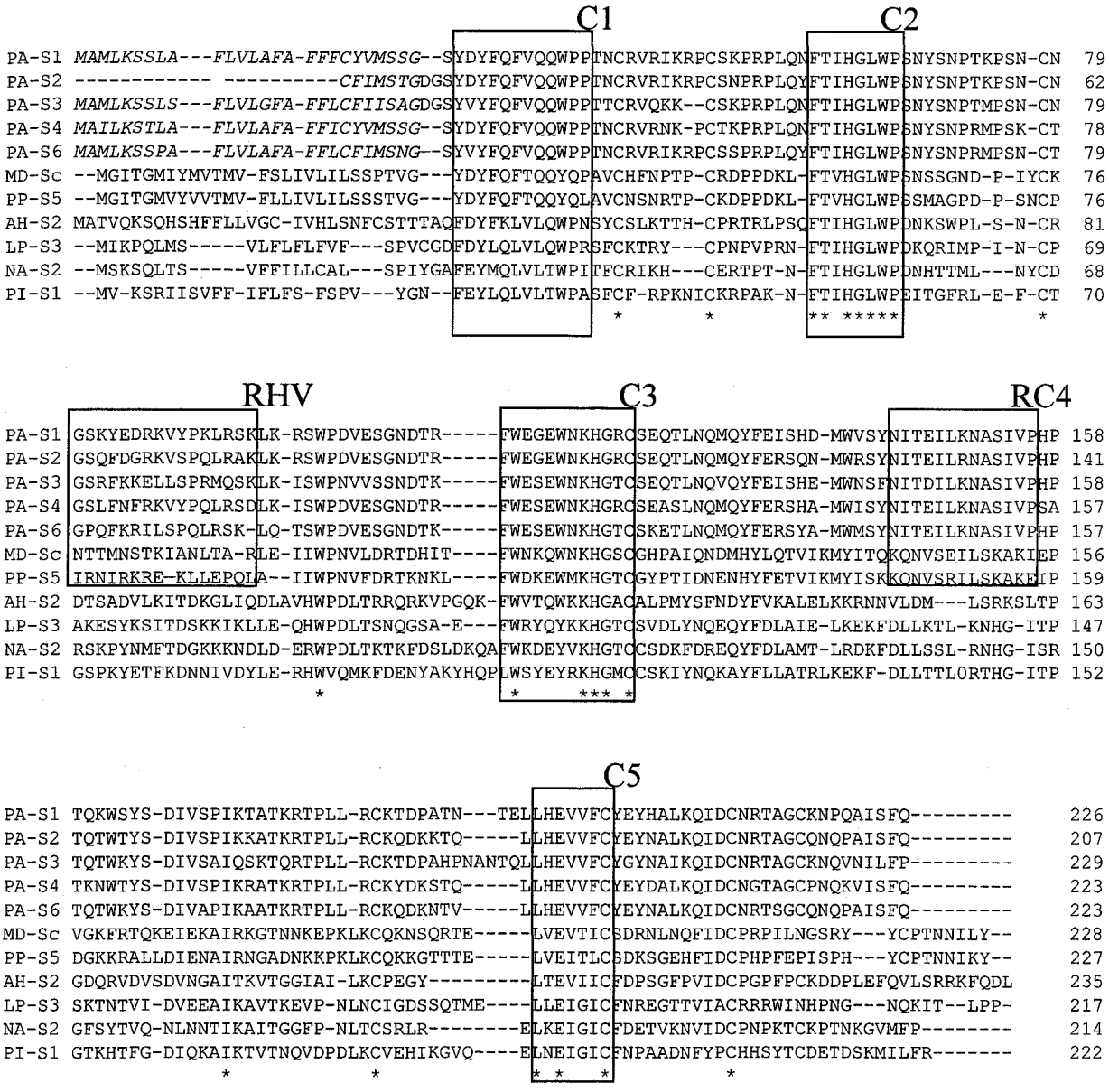

Fig. 1.6 Amino acid sequence alignment of sweet cherry S-RNases and other S-RNases. The alignment was generated by CLUSTAL X (Thompson et al., 1997). Gaps are marked by *dashes*. Signal peptide sequences of sweet cherry S-RNases are indicated by *italics*. Conserved residues in all S-RNases are indicated by asterisks. The five conserved regions, C1, C2, C3, RC4, and C5 and hypervariable region, RHV are shown and boxed. Plant species from which each sequence is derived are denoted by the initials of their scientific names, i.e., PA means *Prunus avium*. Sequence data for the S-RNases included are as follows; S^c of *Malus x domestica* (apple) (Sassa et al., 1996); S^5 of *Pyrus pyrifolia* (Japanese pear) (Sassa et al., 1997); S^2 of *Antirrhinum hispanicum* (Xue et al., 1996); S^2 of *Nicotiana alata* (Anderson et al., 1986); S^1 of *Petunia inflata* (Coleman and Kao, 1992); S^2 of *Lycopersicon pyruvenium* (Royo et al., 1994).

pattern was different from that of the putative pistil S^3-cDNA, suggesting that they were pistil S^2-cDNA. DNA sequencing of these clones confirmed that they contained the sequence encoding the N-terminal amino acid sequence of the putative pistil S^2-protein (Figs. 1.4, 1.6). For 'Satonishiki', 10 independent 3'RACE clones were obtained. When the PCR products obtained with the Pru-C2 and Pru-C5 primers were digested by *Mbo*I, 9 yielded the same restriction fragment pattern as that of the putative pistil S^6-cDNA from the cDNA library. DNA sequencing was performed for the two types of clones and confirmed that they were identical to the putative pistil S^3- and S^6-cDNA clones from the library (Fig. 1.5).

The PCR amplified fragment by the Pru-C2 and Pru-C5 primers of pistil S^3-cDNA was used as probes to screen the cDNA library from 'Rainier' (S^1S^4). About 5000 plaques were screened by these probes and seven positive plaques were converted to pBluescript and PCR amplified by the Pru-C2 and Pru-C5 primers. DNA sequencing of these clones revealed that two kinds of cDNAs were obtained. Both the cDNA clones contained the entire coding sequences (Fig. 1.5). The putative S^1- and S^4-cDNAs include open reading frames of 678 and 669 nucleotides (Fig. 1.5), which encode 226 and 223 amino acid residues (Fig. 1.6), respectively. Deduced amino acid sequences from the cDNAs contained putative signal peptide comprising of approximately 25 amino acid residues at the N-terminal (Fig. 1.6).

The deduced amino acid sequences from the cDNAs encoding S^1-, S^2-, S^3-, S^4-, and S^6-proteins contained two active histidine sites in the C2 and C3 domains of T2/*S* RNase superfamily. Furthermore, they contained the other three conserved domains among rosaceous S-RNases (Ishimizu et al., 1999; Ushijima et al., 1998). In addition to the similarity in the conserved domains, eight cysteine residues conserved among other S-RNases were conserved. Furthermore, the potential N-glycosilation sites that were conserved in *Malus* and *Pyrus* S-RNases (Sassa et al., 1996) were present in sweet cherry within the RC4 domain. These results indicated that the cDNA clones obtained encode S-RNases.

It is interesting that two spots other than the S^2- or S^6-spots were observed in S^2S^3 and S^3S^6 haplotype cultivars (Figs. 1.1 and 1.3). Two different pistil *S*-alleles were present in each cultivar and there should have been two spots, or only one if two different *S*-glycoproteins overlapped on the 2D-PAGE gel. The most reasonable

explanation is that one of the spots is the pistil S^3-allelic product and the other is a non-S-protein. However, N-terminal sequencing suggested that both of them could be pistil S^3-allelic products since the molecules differed only at the N-terminal (Fig. 1.4). It is possible that signal peptidase cleaved the S^3-precursor protein at two different positions and consequently two different mature proteins were produced. The observation that the spots with Asp (acidic amino acid) and Gly (neutral amino acid) at the N-terminal (spots A and A') were located at a more acidic position on the 2D-PAGE gel as compared to the spots without them (spots B and B'), is consistent with the hypothesis that only the N-terminal of the two proteins is different and the rest is the same. Furthermore, the fact that only cDNAs encoding the putative S^3-protein with DG at the N-terminal could be obtained from the cDNA library of 'Satonishiki' and also from 3'RACE clones of 'Satonishiki' and 'Velvet', may support the hypothesis that both spots were pistil S^3-allelic products. Ishimizu et al. (1996) suggested that the signal peptidase in Japanese pear style favored nonpolar side chains at the site of action in the propeptide-mature protein junction, which conforms to the (-3, -1)-rule (von Hijine, 1986). The amino acid sequence around the N-terminal of the mature protein is SAGDGSY and both the Gly-Asp and Gly-Ser bonds could be cleaved without breaking the rule (Fig. 1.6).

Genomic DNA blot analysis

The C2-C5 fragments of putative cDNA clones (Fig. 1.5) of different pistil *S*-alleles seemed to be similar enough to each other to cross-hybridize any of the pistil *S*-alleles even under high stringency wash conditions. One or two bands were observed with all cultivars and with all probes (Fig. 1.7). Cultivars with the same *S*-haplotypes gave the same hybridization signal and each band observed on the blot could be assigned to a pistil *S*-alleles. These results indicated that the cDNAs were associated with *S*-haplotypes of sweet cherry, suggesting that the cDNAs encode S-RNases.

With *Hin*dIII digestion, S^1- and S^3-RNase alleles gave 8.8 kb fragments and S^2-, S^4-, and S^6-RNase alleles gave 5.7 kb fragments (Fig. 1.7). The S^9-RNase allele (S^9-haplotype of some cultivars has been misclassified to S^5-haplotype in previous studies) gave a unique fragment of 3.1 kb. When the C5f-polyA fragment of the putative pistil S^3-cDNA was used as a probe, it specifically hybridized with the pistil

S^3-allele. A single band at 8.8 kb could be detected only with cultivars with S^3-haplotype (Fig. 1.7), although the C5f-polyA fragments of S^1-, S^2-, S^4- and S^6-RNase genes cross-hybridized to all pistil *S*-alleles (data not shown). With *Eco*RI digestion, pistil S^2-, S^3-, S^6-, and S^9-alleles gave unique fragments of 4.4 kb, 13.1 kb, 11.0 kb, and 7.9 kb, respectively (Fig. 1.7). Pistil S^1- and S^4-alleles gave the same fragment of 1.5 kb. When the results obtained with *Hin*dIII and *Eco*RI digestions were combined, pistil S^1- to S^6-alleles could be distinguished from each other. The pistil S^{4m}-allele of 'Compact Stella' gave exactly the same fragment as pistil S^4-allele of other cultivars, which is consistent with the observation that the S^4-RNase was present in the style of 'Compact Stella'.

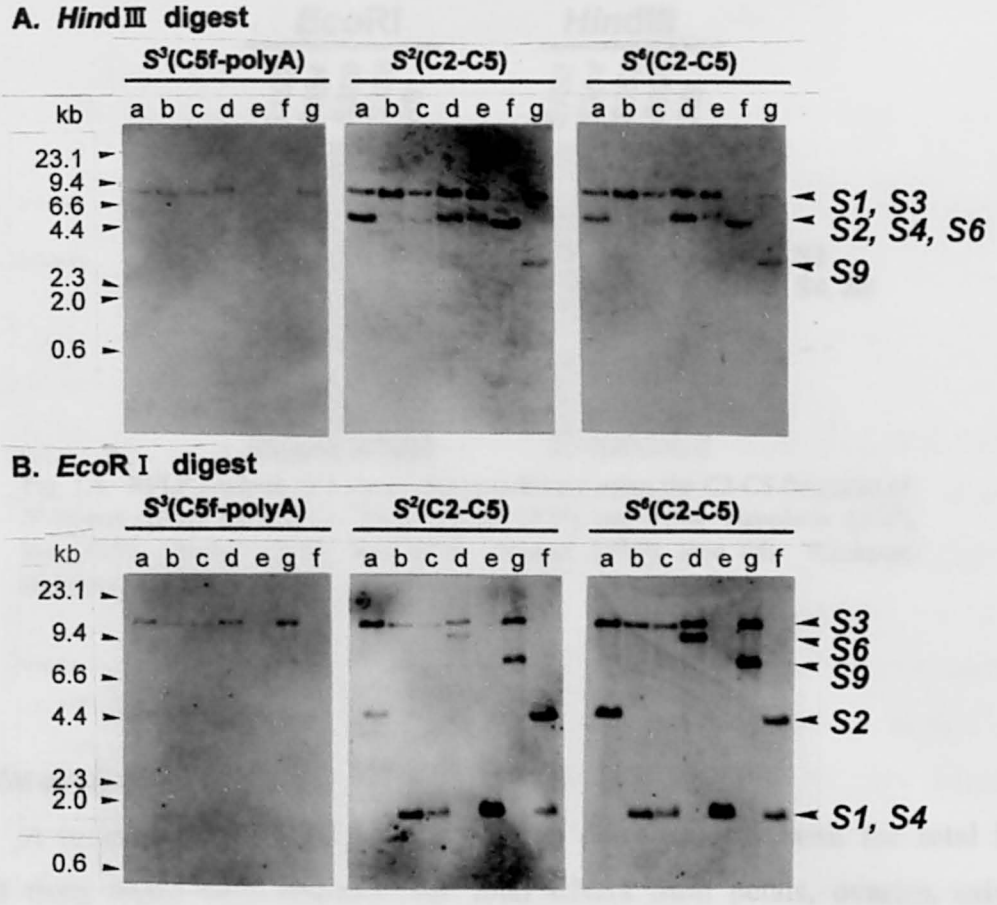

Fig. 1.7. Genomic DNA blot analysis using the C5f-polyA and C2-C5 fragments of the cDNAs encoding S^2, S^3, and S^6-RNases of sweet cherry. (a) Velvet (S^2S^3), (b) Van (S^1S^3), (c) Napoleon (S^3S^4), (d) Satonishiki (S^3S^6), (e) Rainier (S^1S^4), (f) Peggy Rivers (S^2S^4), and (g) Burlat (S^3S^9).

RFLP analysis using additional cultivars confirmed the presence of *S*-haplotype specific polymorphism (Fig. 1.8). Furthermore, the *S*-haplotype of major early ripening sweet cherry cultivar in Japan, 'Takasago (Rockport Bigarreau)', could be determined. 'Takasago' produced 11.0 kb (corresponding to S^6-haplotype) and 1.5 kb (S^1- or S^4-haplotype) bands with *Eco*RI digestion and 5.7 kb (S^2-, S^4- or S^6-haplotype) and 8.8 kb (S^1- or S^3-haplotype) bands with *Hin*dIII digestion. Based on these results, *S*-haplotype of 'Takasago' appears to be S^1S^6, (Fig. 1.8). The DNA-based technique will undoubtedly be more effective to determine *S*-haplotypes of cultivars with unknown *S*-haplotypes than the conventional method for *S*-typing such as controlled pollination tests.

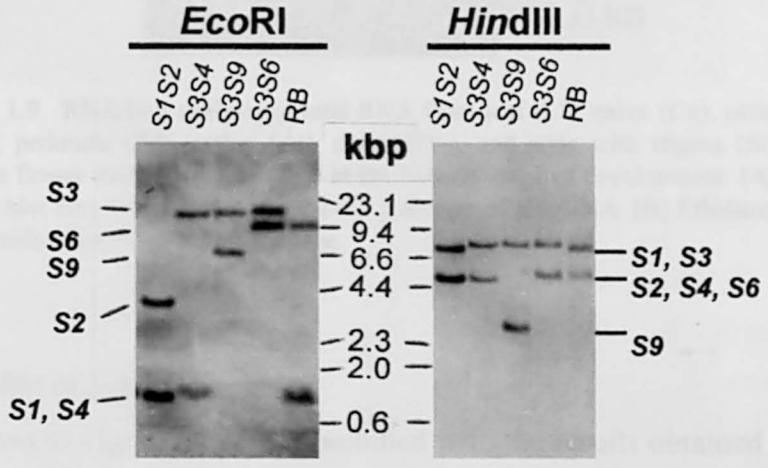

Fig. 1.8. RFLP analysis of 5 sweet cherry cultivars using the C2-C5 fragment of S^2-RNase cDNA. Lane *S1S2*- 'Early Rivers' (S^1S^2), lane *S3S4*- 'Napoleon' (S^3S^4), lane *S3S9*- 'Burlat' (S^3S^9), lane *S3S6*- 'Nanyo' (S^3S^6), lane RB- 'Rockport Bigarreau (Takasago)'.

RNA blot analysis

A hybridization signal at 800 bp was detected only with the total RNA isolated from styles with stigmas. The total RNAs from petals, ovaries, calyxes, anthers, peduncles, and young leaves gave no hybridization signal (Fig. 1.9). There was no difference in the size of the band obtained from the four different probes used, the C2-C5 and the C5f-polyA fragments from the putative pistil S^3- and S^6-cDNAs (data not shown). This result indicated that the genes corresponding to the cDNAs

obtained were specifically transcribed in styles, which is consistent with the expression pattern of other rosaceous S-RNases (Sassa et al., 1996).

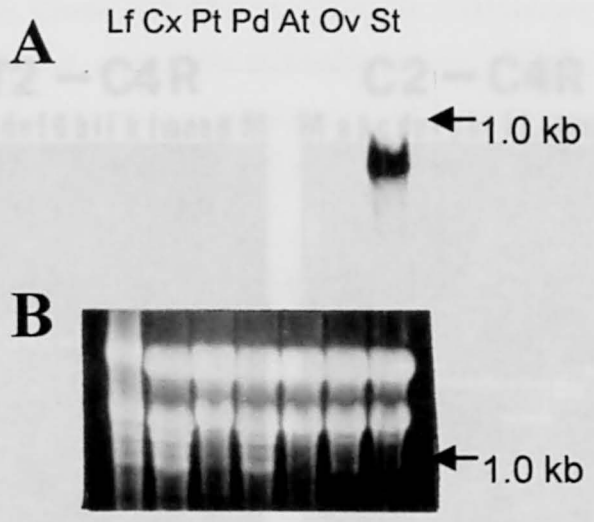

Fig. 1.9. RNA blot analysis of total RNA from leaf (Lf), calyx (Cx), petal (Pt), peduncle (Pd), anther (At), ovary (Ov), and style with stigma (St) from flower buds of 'Satonishiki' at the balloon stage of development. (A) The blot was hybridized to the C2-C5 fragment of S^3-cDNA. (B) Ethidium bromide-stained gel before blotting.

PCR amplification of S-alleles

As shown in Fig. 1.10, when combined with the results obtained from the two primer sets, pistil S^1- to S^6-alleles could be distinguished from each other. With the Pru-T2 and Pru-C4R primers, pistil S^6-, S^1-, and S^4-alleles gave the unique bands of 980, 1220, 1500 bp, respectively, although pistil S^3- and S^9-alleles gave the same bands of 1100 bp. With this primer set, the pistil S^2-band at 2500 bp was faint or sometimes could not be detected. When the Pru-C2 and Pru-C4R primers were used, pistil S^3- and S^9-alleles gave different bands of 750 and 680 bp, respectively, and could be distinguished from each other, although pistil S^1- and S^3-alleles gave the same bands. With this primer set, the pistil S^2-allele could be amplified well and gave the unique band at 2100 bp. Pistil S^4- and S^6-alleles gave unique bands at 1000 and 500 bp, respectively. All the bands amplified with these two primer sets hybridized with the C2-C5 fragment of the pistil S^2-cDNA, which confirmed that they derived from *S*-alleles. As with the DNA blot analysis (Fig. 1.7), the pistil S^{4m}-allele of 'Compact

Stella' gave the same band as pistil S^4-allele of other cultivars. The PCR techniques employed in this study would be useful for a practical *S*-typing system of sweet cherry.

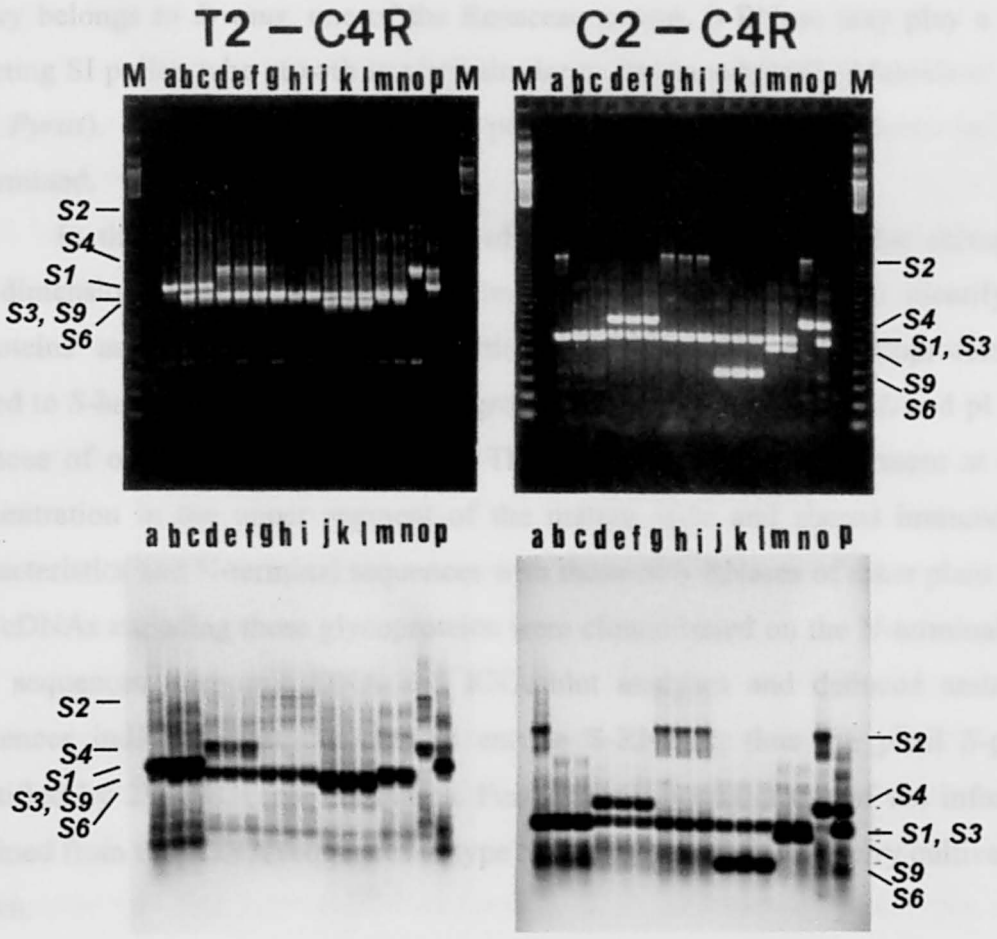

Fig. 1.10. PCR analysis for S-haplotype typing. Genomic DNA was PCR amplified with two primer sets (Pru-T2 + Pru-C4R and Pru-C2 + Pru-C4R), separated on an agarose gel and detected with ethidium bromide staining (upper photograph). The gel was then blotted onto a nylon membrane, and probed and detected with the C2-C5 fragment of S2-cDNA (lower photograph). M: 123 bp DNA ladder. (a) Early Rivers (S^1S^2), (b) Gil Peck (S^1S^3), (c) Van (S^1S^3), (d) Bing (S^3S^4), (e) Napoleon (S^3S^4), (f) Compact Stella (S^3S^{4m}), (g) Velvet (S^2S^3), (h) Victor (S^2S^3), (i) Sue (S^2S^3), (j) Governer Wood (S^3S^6), (k) Nanyo (S^3S^6), (l) Satonishiki (S^3S^6), (m) Burlat (S^3S^9), (n) Moreau (S^3S^9) , (o) Peggy Rivers (S^2S^4), (p) Rainier (S^1S^4).

1.4. Summary

Most sweet cherry cultivars exhibit gametophytic self-incompatibility (Crane and Lawrence, 1929; Crane and Brown, 1937; de Nettancourt, 1977). Since sweet cherry belongs to *Prunus*, one of the Rosaceae genera, S-RNase may play a role in rejecting SI pollen tube growth in pistil similar to that in subfamily Maloideae (*Malus* and *Pyrus*). However, pistil *S*-allelic products of sweet cherry have yet to be determined.

In this chapter, pistil proteins of sweet cherry cultivars were surveyed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to identify pistil *S*-proteins associated with gametophytic self-incompatibility. Glycoprotein spots linked to *S*-haplotypes were found in a group of proteins which had M_r and pI similar to those of other rosaceous S-RNases. These glycoproteins were present at highest concentration in the upper segment of the mature style and shared immunological characteristics and N-terminal sequences with those of S-RNases of other plant species. The cDNAs encoding these glycoproteins were cloned based on the N-terminal amino acid sequences. Genomic DNA and RNA blot analyses and deduced amino acid sequences indicated that the cDNAs encode S-RNases; thus the pistil *S*-proteins identified by 2D-PAGE are S-RNases. Furthermore, potential use of the information obtained from the cDNA sequences to type *S*-haplotypes of sweet cherry cultivars were shown.

Chapter 2
Molecular typing of *S*-haplotypes in sweet cherry (*Prunus avium* L.)

2.1. Introduction

In sweet cherry, identification of pollen-incompatibility groups and assignment of cultivars to the groups are essential for good crop yield. Knight (1969) categorized sweet cherry cultivars into 13 pollen incompatibility groups and Group O, which includes cultivars that are self-incompatible but able to pollinate cultivars in all the other 13 groups. This classification is based on controlled cross-pollination tests and/or pollen tube growth tests (Knight, 1969; Tehrani and Brown, 1992). These tests need to be repeated several times and are time-consuming because pollen tube growth in the style is strongly affected by environmental and physiological factors (de Nettancourt, 1977). Therefore, the *S*-haplotypes of many cultivars including commercially important ones and cultivars in the universal donor (Group O) have yet to be determined.

In Chapter 1, the potential of RFLP for identification of the *S*-haplotypes of cultivars whose S-haplotypes were unknown has been demonstrated. In this chapter, for a reference in future new *S*-haplotype discovery projects, fragment sizes observed in the RFLP analysis, after two endonuclease digestions, were presented for each *S*-haplotype that had been published previously. Furthermore, *S*-haplotypes of three newly released Japanese sweet cherry cultivars and the cultivar classified into Group O were determined by the PCR-based *S*-haplotype typing system. Since different *S*-haplotypes sometimes yield PCR products of the same size, partial sequences of S-RNase genes were determined to develop a more reliable *S*-haplotype identification method of PCR-RFLP analysis.

2.2. Revisiting the *S*-haplotype nomenclature of sweet cherry by RFLP analysis

In this section, RFLP analysis was conducted to characterize 13 sweet cherry *S*-haplotypes that had been published previously since potentially similar sweet cherry *S*-haplotypes have been assigned different nomenclature in several literatures

(Boskovic and Tobutt, 1996; Boskovic et al., 1997; Choi et al., 2000; Knight, 1969; Schmidt and Timmann, 1997; Schmidt et al., 1999; Tehrani and Lay, 1991; Wiersma et al., 2001). Fragment sizes for each *S*-haplotype/restriction enzyme combination are presented to be used as a reference in future *S*-haplotype discovery projects.

2.2.1. Materials and Methods

Plant material

Young leaves of 16 sweet cherry cultivars, 'Burlat', 'Charger', 'Early Rivers', 'Gaucher', 'Gold' 'Guine d'Annonay', 'Hedelfingen', 'Inge', 'Mona', 'Nadino', 'Napoleon', 'NY1625', 'Orleans 171' 'Schneiders', 'Seneca', and 'Valera' (Table 2.1) were used for revisiting the *S*-haplotype nomenclature of sweet cherry cultivars. All leaf samples were lyophilized and stored at – 20 °C until needed for DNA isolation.

Genomic DNA blot analysis

Total DNA was isolated from young leaves using the CTAB method described by Stockinger et al. (1996). In brief, 0.4 g of lyophilized leaves were homogenized and mixed with CTAB solution consisted of 1.25 % CTAB, 0.75 M Tris-HCl (pH 8.5), 375 mM EDTA, 3.75 M NaCl, and 20 µl/ml 2-mercaptoethanol. Then, chloroform/isoamylalcohol were added and centrifuged by 1000 × g for 15 min. The supernatant was removed to a new tube and DNA molecules were precipitated by 5 % CTAB. After purification by ethanol precipitation, DNA was resolved in TE buffer.

Six micrograms of DNA was digested with either *Hin*dIII or *Eco*RI, run on 0.9 % agarose gel for 36 h at 30 V, and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Tokyo) according to Wang et al. (1998). PCR amplified fragments of the S^6-RNase cDNA from sweet cherry were used as the probe. Probes were radiolabelled with ^{32}P-dCTP (DuPont, Boston, USA) using the random primer hexamer-priming method described by Feinberg and Vogelstein (1983). After hybridization at 60 °C for 16 h and high stringency washes (2 × 30 min with 2 × SSC and 1 % SDS followed by 2 × 30 min with 0.2 × SSC and 0.5 % SDS at 60 °C), bound radioactivity resulting from hybridization was exposed to the X-ray film for 4 days and detected.

2.2.2. Results and Discussion

Sixteen sweet cherry cultivars were analyzed by RFLP analyses using *Hin*dIII, or *Eco*RI restriction digestions (Fig. 2.1). The two RFLP analyses gave consistent results, and it was possible to distinguish 13 different putative *S*-haplotypes with each of the four restriction enzymes (Table 2.1).

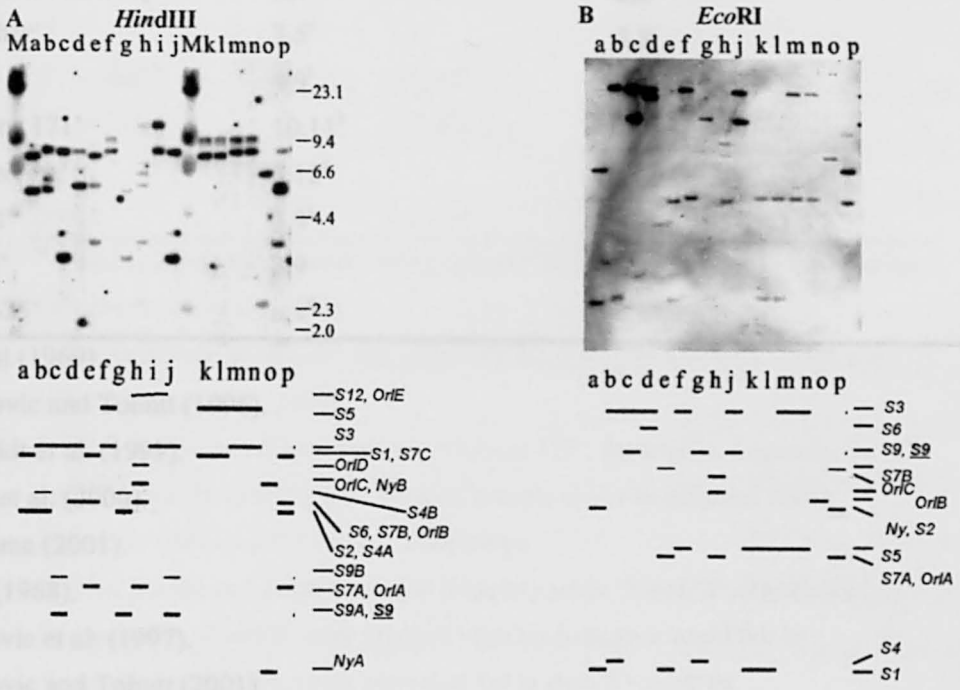

Fig. 2.1. Genomic DNA blot analysis and schematic representation of the genomic blot of 16 sweet cherry cultivars. Six µg of Genomic DNA was digested by A) *Hin*dIII or B) *Eco*RI blotted to membrane and hybridized to the cDNA encoding S^6-RNase. Lambda/*Hin*dIII marker was used for size determination. (a) 'Early Rivers' (S^1S^2), (b) 'Napoleon' (S^3S^4), (c) 'Burlat' (S^3S^9), (d) 'Gold' (S^3S^6), (e) 'Charger' (S^1S^7) (f) 'Gaucher' (S^3S^5), (g) 'Inge' (S^4S^9), (h) 'Orleans 171' ($S^{10}S^{11}$), (i) 'Schneider' (S^3S^{12}), (j) 'Mona' (S^3S^9), (k) 'Seneca' (S^1S^5), (l) 'Valera' (S^1S^5), (m) 'Hedelfingen' (S^3S^5), (n) 'Nadino' (S^3S^5) (o) 'NY1625' (S^uS^v), (p) 'Guigne d'Annonay' (S^2S^7). In figure, Orl represents S^{10} and S^{11} found in 'Orleans 171'. Ny represents S^u and S^v found in 'NY1625'. If more than 1 fragment corresponds to an allele, A is used to designate the smallest fragment, B the next smallest, etc. The fragments corresponding to the S^9 allele from 'Inge' are underlined, whereas the fragments corresponding to the S^9 allele from 'Burlat' are not underlined.

Table 2.1. *S*-haplotypes of 16 sweet cherry cultivars used in this study

Cultivar	*S*-haplotype	Other published nomenclature
'Early Rivers'	1,2[z]	
'Napoleon'	3,4[z]	
'Hedelfingen'	3,5[y,x]	4,5[z], 3,x[w], 3,15[v]
'Nadino'	3,5[x]	3,x[w]
'Seneca'	1,5	1,x[w]
'Valera'	1,5[x,u]	1,x[w], 1,15[v]
'Gold'	3,6[z]	
'Charger'	1,7[t]	
'Guigne d'Annonay'	2,7[s]	2,z[w]
'Gaucher'	3,5[s]	5,8[t]
'Inge'	4,9[t]	
'Orleans 171'	10,11[x]	11[t]
'Schneiders'	3,12[s]	3,13[v], 3,y[w]
'Burlat'	3,9[s]	3,x[x], 4,5[z], 3,5[w, v, r]
'Mona'	3,9	2,14[v]
'NY1625'	u,v[q]	4,x[w]

[z]Knight (1969).

[y]Boskovic and Tobutt (1996).

[x]Schmidt et al. (1999).

[w]Choi et al. (2000).

[v]Wiersma (2001).

[u]Way (1968).

[t]Boskovic et al. (1997).

[s]Boskovic and Tobutt (2001).

[r]Tao et al (1999).

[q] 'NY1625' contained two *S*-haplotypes that did not appear in any of the other sweet cherry cultivars. They have temporarily been named S^u and S^v.

The *S*-haplotypes of 'Early Rivers' (S^1S^2), 'Napoleon' (S^3S^4) and 'Gold' (S^3S^6) have not been questioned in literature since first published (Knight, 1969) (Table 2.1). In the present study, the fragments were separated for a longer period of time on the agarose gel, which enabled S^1 to be distinguished from S^3 while S^2, S^4, and S^6 were distinguished from one another in the *Hin*dIII digest. Similarly, in the *Eco*RI digest, this enabled S^1 to be distinguished from S^4. Each of these five *S*-haplotypes exhibits

Table 2.2. Sizes (kb) of restriction fragments for *S*-haplotypes used in this study

	Size (kb)	
S-haplotype	*Hin*dIII	*Eco*RI
1	8.7	1.5
2	5.6	4.4
3	8.8	13.1
3 'Gaucher' [z]	8.8	13.1
4	5.6, 6.1 [v]	1.8
5	9.4	3.5
6	5.8	11.0
7	3.5, 5.8, 8.7	3.3, 6.0 [v]
9	3.1	7.9
9 'Burlat' [y]	3.1, 4.0 [v]	7.9
10 or 11 [x]	3.5, 5.8, 6.4,	3.3, 5.0, 5.5
12	12.1	--- [u]
u or v [w]	2.5, 6.4	4.8

[z] This haplotype in 'Gaucher' was originally thought to be a unique *S*-haplotype (S^8) (Boskovic et al., 1997).

[y] This haplotype in 'Burlat' was originally thought to be a unique *S*-haplotype (S^x) (Schemidt et al., 1999).

[x] These are the two *S*-haplotypes in 'Orleans 171'. Restriction fragments for S^{10} and S^{11} were grouped together because it could not be determined which fragments corresponded to each *S*-haplotype

[w] These are the two putative unique *S*-haplotypes in 'NY1625'. Restriction fragments for S^u and S^v were grouped together because it could not be determined which fragments corresponded to each *S*-haplotype

[v] Denotes bands that were very faint

[u] Missing data.

just one fragment with the exception of the S^4, which exhibit two fragments following *Hin*dIII (Table 2.2, Fig. 2.1).

As reported by Boskovic et al. (1997), 'Charger' (S^1S^7) and 'Inge' (S^4S^9) each exhibit one new *S*-haplotype. These *S*-haplotypes, called S^7 and S^9, displayed from one to three unique fragments per haplotype following Southern hybridization (Table 2.2, Fig. 2.1). The presence of two unique *S*-haplotypes in 'Orleans 171' ($S^{10}S^{11}$) also agrees with that of Boskovic et al. (1997). RFLP analysis of 'Orleans 171' produced

either three or five fragments in the *Eco*RI or *Hin*dIII digest, respectively (Table 2.2, Fig. 2.1) and the fragment patterns did not match that of any known *S*-haplotypes. It could not be determined which RFLP fragments represent the S^{10} versus the S^{11} since differential cultivars, such as S^3S^{10} and S^3S^{11} are not available. These results support the conclusion that 'Orleans 171' contains two unique *S*-haplotypes. However, none of the two restriction digests were able to detect a difference between S^3 and S^8 (Table 2.2, Fig. 2.1). As for 'Gaucher', recent cloning and sequencing of the S^8-haplotype has shown that the sequence for the S^3- and S^8-RNases are identical (Sonneveld et al., 2001). Therefore, the *S*-haplotype designation for 'Gaucher' should be S^3S^5 rather than S^5S^8.

The *S*-haplotypes of the other cultivars in Table 2.2 have been more difficult to determine and much of this difficulty can be traced to the initial misclassification of 'Hedelfingen' and 'Burlat' (Knight, 1969). There is no doubt that 'Hedelfingen' and 'Burlat' have S^3-haplotype. However, Choi et al. (2000) called the other *S*-haplotype in 'Hedelfingen' S^{α} and Wiersma et al. (2001) have named this S^{15}. Both groups of researchers called the other *S*-haplotype in 'Burlat' S^5. Nevertheless, both Boskovic et al. (1997) and Schmidt et al. (1999) called the *S*-haplotype in 'Hedelfingen' S^5 prior to either of these publications. Therefore, S^5 should be adopted as the standard nomenclature for the haplotype present in 'Hedelfingen' and also in 'Nadino' (S^3S^5), 'Seneca' (S^1S^5), 'Valera' (S^1S^5), and 'Gaucher' (S^3S^5) (Table 2.1, Fig. 2.1). This S^5-haplotype exhibited just one fragment when digested with any of the two restriction enzymes (Table 2.2, Fig. 2.1).

The unique *S*-haplotype present in 'Burlat' that was called S^5 (Choi et al., 2000; Wiersma et al., 2001, see Chapter 1) and S^{α} (Schmidt et al., 1999) should be renamed. Recently, this *S*-haplotype has been sequenced, and found to have an identical sequence to the S^9-haplotype found in 'Inge' (Sonneveld et al., 2001). Therefore, the *S*-haplotype nomenclature for 'Burlat' should be S^3S^9. The RFLP profiles of the S^9-haplotype in 'Burlat' are similar to the profiles of the S^9-haplotype from 'Inge' following digestion with *Hin*dIII and *Eco*RI, with the only differences being the presence of extra faint bands in the 'Burlat' S^9-haplotype when digested with *Hin*dIII (Table 2.2, Fig. 2.1). This can be explained by differential length of exposure or differing amounts of DNA in the digestion reaction.

'Mona' used in this research has the same haplotypes as 'Burlat' (S^3S^9) (Fig. 2.1). This contradicts the finding by Wiersma et al. (2001) that the *S*-haplotype of 'Mona' is S^2S^{14}. The probable explanation is that the 'Mona' trees from which the leaves were collected for study (the USDA Clonal Repository, Davis, Calif. and Vineland, Ontario, Canada, respectively) were not the same cultivar.

Choi et al. (2000) determined that 'Guine d'Annonay' contained an *S*-haplotype that differed from any previously reported *S*-haplotype and thus named it S^e. The *Hin*dIII and *Eco*RI RFLP analyses all suggest that the S^e-haplotype in 'Guine d'Annonay' is the same as the S^7-haplotype in 'Charger' (Fig. 2.1). Therefore, we propose that the actual *S*-haplotype of 'Guine d'Annonay' should be S^2S^7 (Table 2.1).

'Schneiders' has been reported to contain an additional unique *S*-haplotype, named S^y by Choi et al. (2000), S^{13} by Wiersma et al. (2001), and S^{12} by Boskovic and Tobutt (2001). In order to remain consistent with the European nomenclature, we suggest retaining the haplotype of S^3S^{12} for 'Schneiders'.

The RFLP patterns from the *Hin*dIII suggest that the selection 'NY1625' contains two *S*-haplotypes represented by one fragment each that are not found in any other cultivar (Table 2.2, Fig. 2.1). Therefore, these two *S*-haplotypes are temporarily named S^u and S^v until crossing data confirms that they are indeed unique *S*-haplotypes.

The potential of RFLP for discovery and identification of new *S*-haplotypes has been demonstrated based on their unique banding patterns after digestion with any of the two restriction enzymes used in the present study. Therefore, this RFLP analysis enables us to determine if the *S*-haplotype is likely a new haplotype, or if it matches an already existing *S*-haplotype by comparing its fragment sizes with those presented in Table 2.2.

2.3. Genomic structure of S-RNases and *S*-typing by PCR-RFLP analysis

PCR typing system facilitates determination of the *S*-haplotypes of new cultivars and discovery of new *S*-haplotypes or new incompatibility groups because PCR analysis is much simpler than genomic DNA blot analysis. Nevertheless, different *S*-haplotypes sometimes yield PCR products of the same size, which potentially causes errors in *S*-typing. Thus, partial sequences of S-RNase genes were determined to develop a more reliable *S*-haplotype identification method of PCR-RFLP. Using this system, the *S*-haplotypes were determined for three newly released Japanese sweet cherry cultivars ('Koukanishiki', 'Benisayaka' and 'Benishuho') (Sato et al., 1993) and the British cultivar 'Merton Glory' that was classified as a universal donor.

2.3.1. Materials and Methods

Plant material and isolation of genomic DNA

Total DNA was isolated from young leaves of three newly released Japanese sweet cherry cultivars, 'Benisayaka', 'Benishuho' and 'Koukanishiki', and one cultivar that was classified as a universal donor (Knight, 1969), 'Merton Glory', by the CTAB method (Doyle and Doyle, 1987), combined with PEG precipitation (Mak and Ho, 1993) as described in Chapter 1, and used for PCR analysis. As references, total DNAs of ten self-incompatible sweet cherry cultivars with known *S*-haplotypes, 'Burlat' (S^3S^9), 'Early Rivers' (S^1S^2), 'Governor Wood' (S^3S^6), 'Nanyo' (S^3S^6), 'Napoleon' (S^3S^4), 'Peggy Rivers' (S^2S^4), 'Van' (S^1S^3) 'Velvet' (S^2S^3), 'Rainier' (S^1S^4), 'Satonishiki' (S^3S^6), were also isolated and used for PCR analysis.

PCR analysis

Two sets of primers that were designed from DNA sequences encoding the signal peptide (Pru-T2) and two conserved domains (Pru-C2 and Pru-C4R) of sweet cherry S-RNases, as described in Chapter 1, were used for PCR. The PCR reaction mixture and PCR program were identical to those described in Chapter 1. After PCR, PCR mixture was run on 1.5 % agarose gel and DNA bands were visualized by ethydium bromide staining.

Cloning and DNA sequencing of PCR-amplified fragments

PCR products from 'Satonishiki (S^3S^6)' and 'Rainier (S^1S^4)' were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, WI) and their sequences were determined with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo) and the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Tokyo).

Digestion of PCR-amplified fragments with restriction enzymes

PCR products from 4 cultivars, 'Benisayaka', 'Benishuho', 'Koukanishiki' and 'Merton Glory', were digested by *Ava*I, *Apa*LI, *Nru*I, or *Ban*II. As references, PCR products of the ten cultivars with known *S*-haplotypes were also digested by the four restriction enzymes. The digestion mixture contained 3 μl of PCR products and 10 U of restriction enzyme in an appropriate restriction buffer in a 10 μl reaction volume. After digestion for 2 h at 37 °C, digests of 4 cultivars along with those of 10 cultivars with known *S*-haplotypes were run on 1.5 % agarose gel and DNA bands were visualized by ethydium bromide staining.

2.3.2. Results and Discussion

PCR analysis

The expected size of PCR products as described in Chapter 1 was obtained for S^1-, S^2-, S^3-, S^4-, S^6- and S^9-haplotypes by both primer sets used (Fig. 2.2). Two *S*-haplotypes were identified for each cultivar. For 'Koukanishiki', two bands corresponding to S^1- and S^3-haplotypes were obtained with both primer sets (Fig. 2.2). When the Pru-T2 and Pru-C4R primer set was used, bands of 1220 bp and 1100 bp, which corresponded to S^1- and S^3-haplotypes, respectively, were obtained (Fig. 2.2). With the Pru-C2 and Pru-C4R primer set, a single band of 750 bp, which corresponded to two *S*-haplotypes, S^1- and S^3-haplotypes, was obtained (Fig. 2.2). Similarly, 'Benisayaka' yielded two bands corresponding to S^1- and S^6-haplotypes with both primer sets (Fig. 2.2). 'Benishuho' and 'Merton Glory' gave exactly the same banding pattern with two bands corresponding to S^4 and S^6-haplotypes for both primer sets (Fig.

2.2). From these observations, the *S*-haplotypes of 'Koukanishiki', 'Benisayaka', 'Benishuho', and 'Merton Glory' were suggested to be S^1S^3, S^1S^6, S^4S^6, and S^4S^6, respectively.

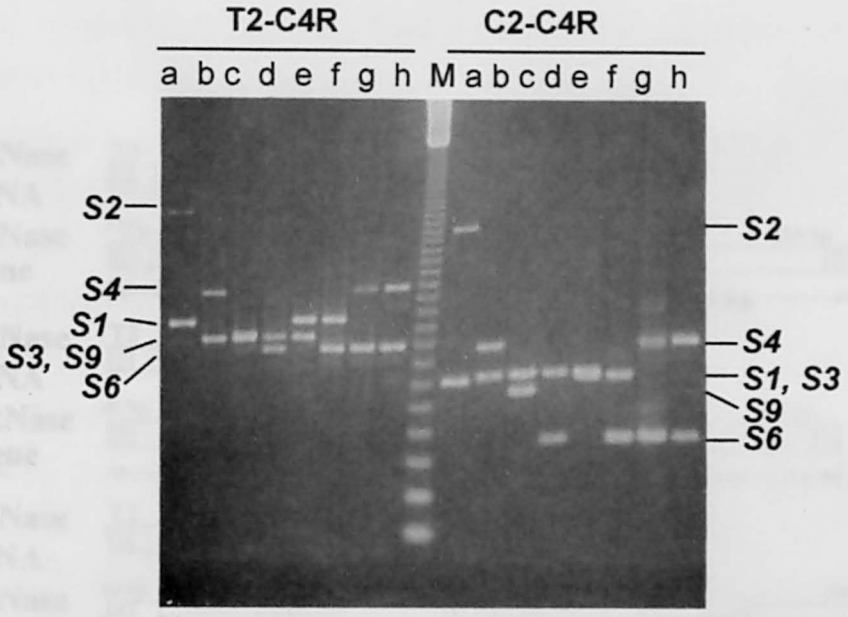

Fig. 2.2. PCR analysis of sweet cherry cultivars with the Pru-T2 and Pru-C4R or the Pru-C2 and Pru-C4R primer sets. Lane M: 123 bp DNA ladder, lane a: 'Early Rivers' (S^1S^2), lane b: 'Napoleon' (S^3S^4), lane c: 'Burlat' (S^3S^9), lane d: 'Satonishiki' (S^3S^6), lane e: 'Koukanishiki', lane f: 'Benisayaka', lane g: 'Benishuho', and lane h: 'Merton Glory'.

Characterization and DNA sequence of genomic DNA encoding S-RNases

DNA sequences were determined for the fragments obtained using the Pru-T2 and Pru-C4R primers from S^3- and S^6-RNase genes of 'Satonishiki (S^3S^6)' (DDBJ/EMBL/GenBank AB031816 and AB031818) and the S^1- and S^4-RNase genes of 'Rainier (S^1S^4)' (DDBJ/EMBL/GenBank AB031815 and AB031817). The size of the

PCR products from S^1-, S^3-, S^4-, and S^6-RNase genes were 1199, 1061, 1463, and 970 bp, respectively (Fig. 2.3). The exons of the genomic DNA sequences of S^1-, S^3-, S^4-, and S^6-RNase genes were completely consistent with the corresponding coding regions of the cDNA sequences of S^1-, S^3-, S^4-, and S^6-RNase genes, respectively. Two introns were inserted into all four S-RNase genes investigated (Fig. 2.3). All the other S-RNase genes reported so far had only a single intron located within a region of high

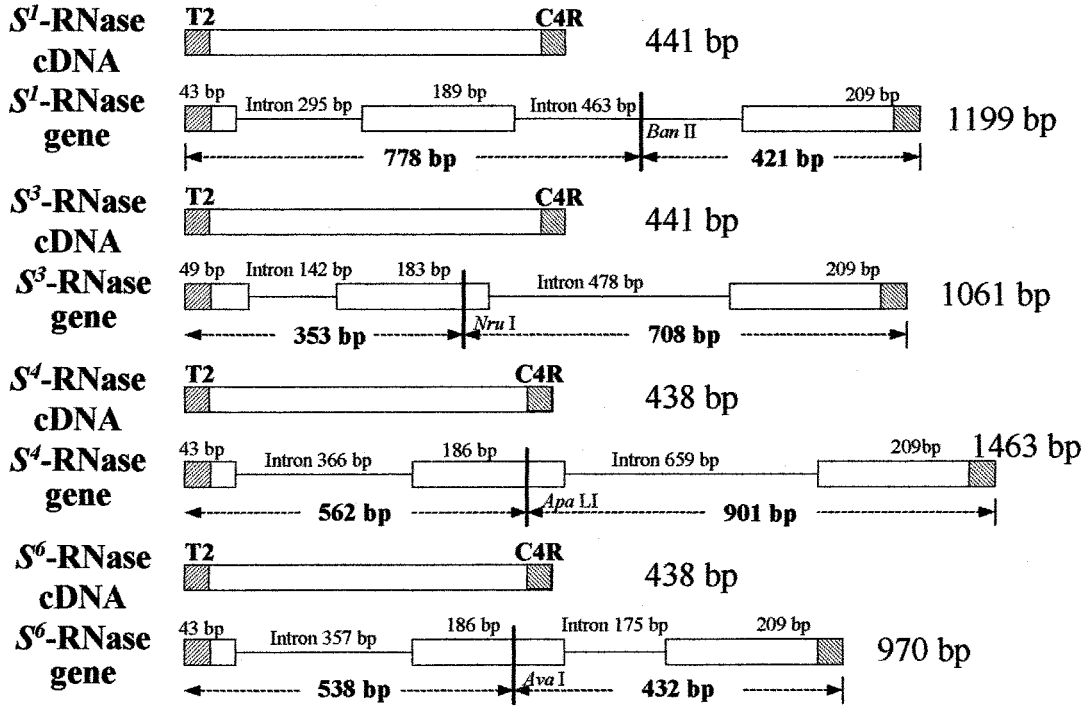

Fig. 2.3. Schematic representation of the PCR amplified product of genomic DNA structures for the four S-RNase genes (S^1, S^3, S^4 and S^6) and their corresponding cDNAs in sweet cherry. Location of the primers used in PCR analysis, length of PCR-amplified fragments of genomic DNAs and cDNAs, and of introns, exons and PCR-amplified fragments after digestion by appropriate restriction enzymes are indicated. Introns were represented by the bars between two boxes representing exons. The cleavage sites by four restriction enzymes (*Ava* I, *Apa* LI, *Ban* II and *Nru* I) are indicated by vertical lines.

interallelic variability, HVa for Solanaceous and RHV for Rosaceous S-RNases (Chung et al., 1995; Coleman and Kao, 1992; Ioerger et al., 1991; Kaufmann et al., 1991; Matton et al., 1995; Ushijima et al., 1998a). In sweet cherry S-RNase genes, the first introns were 295, 142, 366, and 357 bp in length for the pistil S^1-, S^3-, S^4-, and S^6-alleles, respectively (Fig. 2.3). They were inserted within the sequence corresponding to the junction between signal peptides and mature proteins. The second introns were 463, 478, 659, and 175 bp in length for the pistil S^1-, S^3-, S^4-, and S^6-alleles, respectively (Fig. 2.3). They were found in the rosaceous hypervariable region (RHV) (Ushijima et al., 1998b) of S-RNases, as in other rosaceous S-RNases (Ma and Oliveira, 2001; Tamura et al., 2000; Ushijima et al., 1998a).

Within the PCR-amplified partial sequences of S^1-, S^3-, S^4- and S^6-RNase genes, *S*-allele-specific cleavage sites of the restriction enzymes were found (Fig. 2.4). The recognition sites of *Ban*II, *Nru*I, *Apa*LI and *Ava*I were unique in the fragments from the S^1-, S^3-, S^4- and S^6-alleles, respectively (Fig. 2.4). For example, when the 1199 bp of S^1-RNase gene obtained with Pru-T2 and Pru-C4R primers was digested with *Ban* II, it produced 778 and 421 bp fragments while the other three S-RNase genes were not digested by *Ban* II (Fig. 2.4). Likewise, the 1061, 1463 and 970 bp of S^3-, S^4- and S^6-RNase genes digested by *Nru* I, *Apa* LI and *Ava* I produced 708 and 353 bp, 901 and 562 bp, and 538 and 432 bp fragments, respectively (Fig. 2.4). RFLP analysis of PCR products with these enzymes would provide more reliable information for identification of these four S-RNase genes.

Identification of the S-haplotypes of four cultivars through PCR-RFLP analysis

The Pru-T2 and Pru-C4R fragments that corresponded to the S^1-RNase gene of 'Koukanishiki' and 'Benisayaka' could be digested by *Ban* II to produce two fragments of 778 and 421 bp, whereas the other three enzymes, *Apa*LI, *Ava*I, and *Nru*I, did not cut the fragments (Fig. 2.5AB). *Nru*I digestion of the fragment corresponding to the S^3-RNase gene of 'Koukanishiki' yielded two fragments of 708 and 353 bp but the other three enzymes did not cut the fragment (Fig. 2.5A). Similarly, the fragment corresponding to the S^4-RNase gene of 'Benishuho' and 'Merton Glory' was digested by *Apa*LI and produced fragments of 901 and 562 bp but the other three enzymes did not cut the fragment (Fig. 2.5CD). Also, the S^6-fragment of 'Benisayaka', 'Benishuho',

and 'Merton Glory' digested by *Ava*I produced fragments of 538 and 432 bp (Fig. 2.5 BCD). These results confirmed that *S*-haplotypes of 'Koukanishiki', 'Benisayaka', 'Benishuho', and 'Merton Glory' were S^1S^3, S^1S^6, S^4S^6, and S^4S^6, respectively, as has

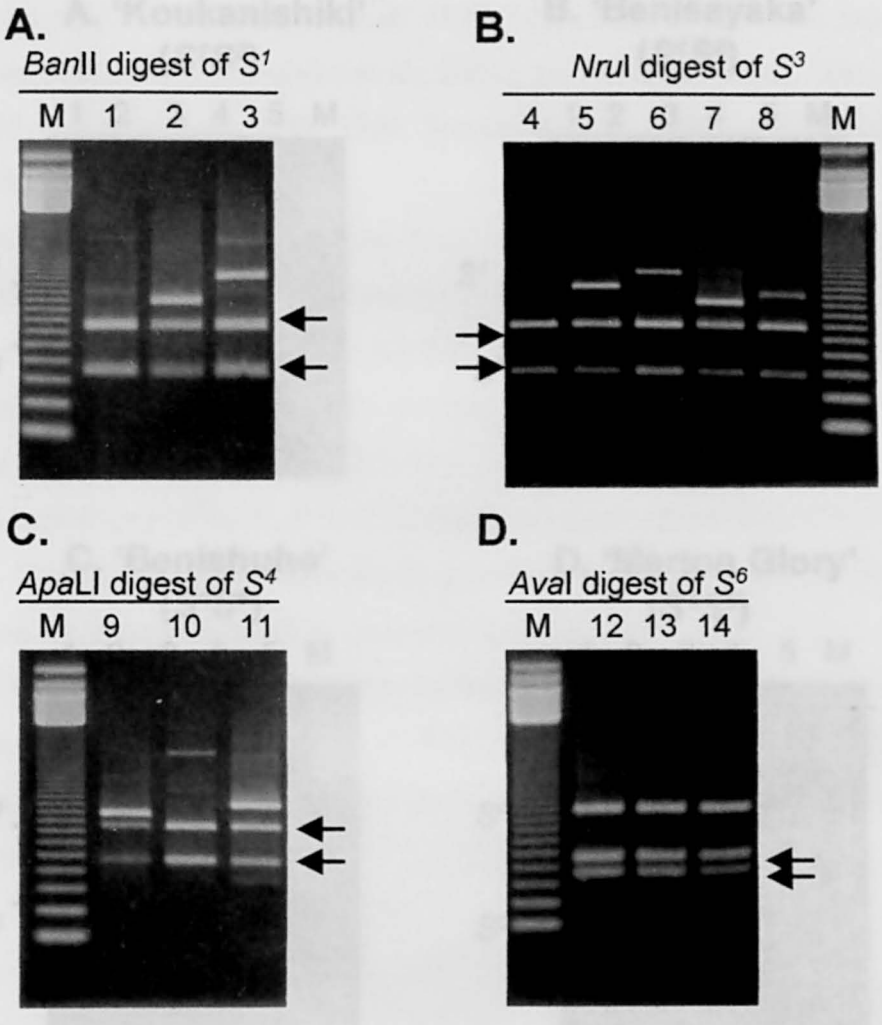

Fig. 2.4. Digestion of PCR products using Pru-T2 and Pru-C4R primers by restriction enzymes. Lane 1: 'Early Rivers' (S^1S^2), lane 2 and 5: 'Van' (S^1S^3), lane 3 and 11: 'Rainier' (S^1S^4), lane 4: 'Velvet' (S^2S^3), lane 6 and 9: 'Napoleon' (S^3S^4), lane 7 and 12: 'Satonishiki' (S^3S^6), lane 8: 'Burlat' (S^3S^9), lane 10: 'Peggy Rivers' (S^2S^4), lane 13: 'Governor Wood' (S^3S^6), lane 14: 'Nanyo' (S^3S^6), and lane M: 123 bp DNA ladder. Two digested fragments by each restriction enzyme are indicated with *arrows*. Note that the bands from S^2-haplotype with the size of 2500 bp were sometimes very faint.

been suggested by the size of the PCR products (Fig. 2.5). Since different *S*-haplotypes sometimes yield PCR products of the same size, RFLP analysis of the PCR products gives more reliable information for *S*-typing.

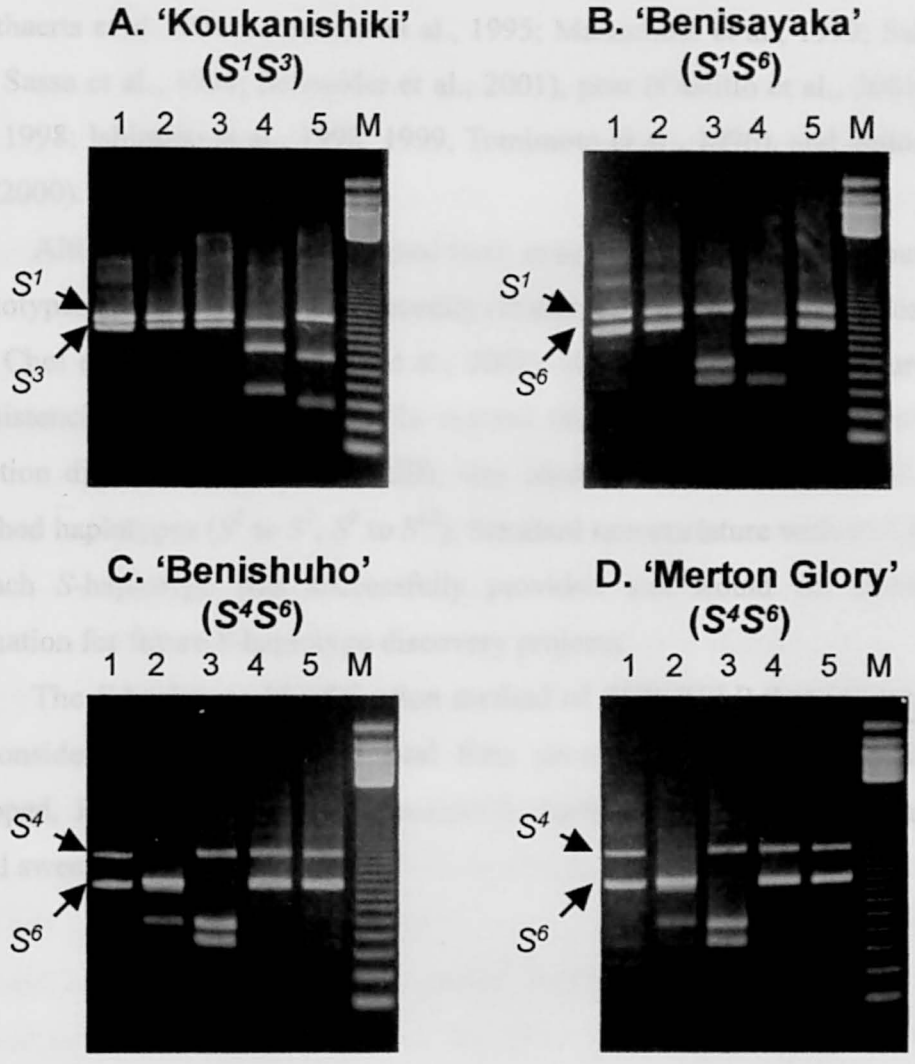

Fig. 2.5. *S*-haplotype typing of 4 cultivars, 'Koukanishiki' (A), 'Benisayaka' (B), 'Benishuho' (C), and 'Merton Glory' (D) by PCR-RFLP analysis. PCR products obtained using Pru-T2 and Pru-C4R primers were digested with: Lane 1: uncut, lane 2: *Apa* LI, lane 3: *Ava* I, lane 4: *Ban* II, and lane 5: *Nru* I. Lane M: 123 bp ladder.

2.4. Summary

Correct assignment of *S*-haplotypes in sweet cherry is important to assure fruit set in field planting and breeding crosses. In this chapter, it was demonstrated that DNA-based technology for the *S*-typing was practicable as is the case with apple (Broothaerts et al., 2001; Janssens et al., 1995; Matsumoto et al., 1999; Sakurai et al., 1997; Sassa et al., 1994; Schneider et al., 2001), pear (Castillo et al., 2001; Hiratsuka et al., 1998; Ishimizu et al., 1998, 1999, Tomimoto et al., 1996), and almond (Tamura et al., 2000).

Although six *S*-haplotypes had been assigned previously, numerous additional *S*-haplotypes have been identified recently (Boskovic and Tobutt, 2001; Boskovic et al., 1997; Choi et al., 2000; Wiersma et al., 2001). However, the nomenclature contained inconsistencies and redundancies. To resolve this conflict, RFLP analysis with two restriction digests, *Eco*RI and *Hin*dIII, was conducted to differentiate 11 previously published haplotypes (S^1 to S^7, S^9 to S^{12}). Standard nomenclature with RFLP band sizes for each *S*-haplotype was successfully provided and would be useful reference information for future *S*-haplotype discovery projects.

The *S*-haplotype identification method of PCR-RFLP that is a much simpler and considered to be more practical than genomic DNA blot analysis was also developed. PCR-RFLP analysis successfully enabled us to identify *S*-haplotypes of several sweet cherry cultivars.

Chapter 3
Genetic control of self-incompatibility and self-compatibility in tetraploid sour cherry (*Prunus cerasus* L.)

3.1. Introduction

One interesting aspect of gametophytic self incompatibility is that it commonly 'breaks down' as a result of polyploidy resulting in self-compatible individuals. This breakdown of gametophytic self-incompatibility in polyploid plants with self-incompatible diploid relatives has been documented in *Lycopersicon* Mill. (Chawla et al., 1997; de Nettancourt et al., 1974), *Nicotiana* L. (Pandy, 1968), *Petunia* Juss. (Entani et al., 1999; Stout and Chandler, 1942), *Solanum* L. (Livermore and Johnstone, 1940), *Trifolium* L. (Brewbaker, 1954), *Rosa* (Ueda and Akimoto, 2001), and *Pyrus* (Crane and Lewis, 1942). To explain this phenomenon, Lewis (1947) proposed that pollen containing two different *S*-haplotypes loses its self-incompatible phenotype resulting in self-compatible polyploid individuals. Evidence obtained from recent research in solanaceous species supports this theory (Chawla et al. 1997; Entani et al. 1999; Golz et al. 1999, 2001; Luu et al. 2001).

In contrast, the gametophytic self-incompatible diploid sweet cherry (*Prunus avium* L., 2n=2x=16) and the tetraploid sour cherry (*P. cerasus* L., 2n=4x=32) represent a natural diploid - tetraploid series where the tetraploid individuals can be either self-incompatible or self-compatible (Lansari and Iezzoni, 1990; Redalen, 1984a, b). Sweet cherry and the tetraploid ground cherry (*P. fruticosa* Pall., 2n=4x=32) are believed to be the ancestral species that gave rise to sour cherry many times via unreduced gametes from sweet cherry (Olden and Nybom 1968; Iezzoni and Hancock 1984; Brettin et al. 2000). Although the vast majority of sour cherry cultivars are self-compatible, numerous self-incompatible cultivars exist in Eastern Europe, the center of diversity (Lech and Tylus 1983; Redalen 1984a, b; Lansari and Iezzoni 1990; Iezzoni et al. 1990).

Sour cherry is one of the major fruit tree species of *Prunus*, especially in European countries and USA (Iezzoni, 1990). Since any successful new sour cherry cultivar would have to be self-compatible to avoid the production problems associated

with providing pollinator trees, it would be very important to determine the genetic basis of self-incompatibility and self-compatibility in sour cherry to increase the likelihood of obtaining self-compatible progeny in a sour cherry breeding program. In this chapter, genetic control of self-incompatibility and self-compatibility in tetraploid sour cherry was investigated with respect to S-RNases identified in this study.

3.2. Identification and characterization of S-RNases in tetraploid sour cherry (*Prunus cerasus* L.)

In contrast to sweet cherry (*Prunus avium* L.), inheritance of self-incompatibility in sour cherry (*P. cerasus* L.) has not been determined and no *S*-haplotype designations have been proposed. Sour cherry is thought to have resulted from hybridization between sweet cherry and the tetraploid ground cherry (*P. fruticosa* Pall). Due to the evolutionary relatedness of sweet and sour cherries, and the potentially ongoing gene flow between the two species, sour cherry is likely to possess *S*-haplotype encoding pistil S-RNases. To date, no studies of S-RNases in sour cherry have been reported. In this section, evidence is presented that both self-incompatible and self-compatible sour cherry selections have stylar RNases, some of which appear to be similar to the S-RNases already cloned from sweet cherry. Furthermore, inter-specific crosses revealed that S-RNases of sour cherry styles are functional to reject pollen that has the same *S*-haplotypes.

3.2.1. Materials and Methods

Plant material

Thirteen sour cherry cultivars and selections growing at the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, Mich., were used for this research: 'Cigany 59', 'Crisana', 'Erdi Botermo', 'Erdi Nagygyumolcsu', 'Favorit', 'Montmorency', 'Pandy 38', 'Pandy 114', 'Rheinische Schattenmorelle', 'Surefire', 'Tschernokorka', 'Ujfehertoi Furtos', and MSU seedling selection I 20 (36). Fifteen sweet cherry cultivars, 'Bing', 'Burlat', 'Charger', 'Early Rivers', 'Gold', 'Goucher', 'Guigne d' Annonay', 'Hedelfingen', 'Inge', 'Mona', 'Orleans 171',

'Rainier', 'Satonishiki', 'Schmidt', and 'Schneiders', were used to provide standards for the previously reported *S*-haplotypes, S^1 - S^7, S^9 - S^{12} or used for the pollen tube growth assays (Table 3.1).

Table 3.1. Sweet cherry cultivars used as *S*-haplotype standards and their *S*-haplotypes

Cultivar	*S*-haplotype
Early Rivers	S^1S^2
Bing	S^3S^4
Gaucher, Hedelfingen	S^3S^5
Gold, Satonishiki	S^3S^6
Charger	S^1S^7
Guigne d'Annonay	S^2S^7
Burlat, Mona	S^3S^9
Inge	S^4S^9
Orleans 171	$S^{10}S^{11}$
Schneiders	S^3S^{12}
Rainier	S^1S^4
Schmidt	S^2S^4

Pollen tube growth assays

Self-pollinated pollen tube growth from the sour cherry cultivars and selections was observed. In addition, eight interspecific crosses were performed and pollen tube growth was observed. Styles from each of the sour cherry cultivars ('Crisana' and 'Tschernokorka') were pollinated with pollen from the sweet cherry cultivars, 'Satonishiki' or 'Rainier'. Styles from the sweet cherry cultivars ('Rainier' and 'Schmidt') were pollinated with 'Crisana' and 'Tschernokorka' pollen. Pollination tests were performed based on the method by Lansari and Iezzoni (1990) but with substantial modifications. Pollen from newly opened flowers was collected from each of the pollen parents and from a bulk consisting of several cultivars (out-cross pollen). For each of the pistil parents, a branch with flowers at the balloon stage was brought into the lab (25 °C) and twenty flowers were emasculated. All other flowers were removed. Ten emasculated flowers were hand pollinated when receptive (24 h after

emasculation) with each of the pollen sources. The other 10 flowers were pollinated with out-cross pollen. The pollinated pistils were collected 72 h after pollination and immersed in fixing solution [(1:3:1, chloroform : 95 % ethanol : glacial acetic acid) (v/v)] for 24 h, transferred to 100 % ethanol, and stored at 4 °C until used. The pistils were washed thoroughly under running tap water and incubated in 10 N NaOH for 5 to 6 h to soften the tissues. The pistils were then soaked in 0.1 % aniline blue solution with 33 mM K_3PO_4 for 1 h. Pollen tubes were observed by ultraviolet fluorescent microscopy (BX60, Olympus, Tokyo, Japan).

Protein assay

Styles with stigmas were dissected from flower buds of 11 sour cherry cultivars and selections except for 'Cigany 59' and 'Pandy 38' at the balloon stage of development, frozen immediately in liquid nitrogen, and lyophilized. Acetone powder was prepared from the lyophilized samples as described in Chapter 1 and used for protein assay. Crude extracts from the acetone powder were subjected to 2D-PAGE using NEPHGE in the first dimension and SDS-PAGE in the second dimension (Tao et al., 1997). After electrophoresis, proteins in the gel were detected by silver staining using Sil-Best Stain for Protein/PAGE (Nacalai tesque, Kyoto, Japan).

For 'Rheinische Schattenmorelle' and 'Erdi Botermo', the proteins separated by 2D-PAGE were electroblotted onto a PVDF membrane as described in Chapter 1 and glycoproteins were detected with biotin-conjugated Con A and horseradish peroxidase-conjugated streptavidin. Peroxidase activity on the membrane was visualized using 4-chloro-1-naphthol. Immunodetection of proteins electroblotted onto PVDF membrane was also conducted for 'Rheinische Schattenmorelle' and 'Erdi Botermo' using the rabbit anti-S^c-serum prepared against recombinant S^c-RNase of almond (Ushijima et al., 2001). In addition, for 'Erdi Botermo', after the proteins were blotted onto the PVDF membrane and detected by Coomassie Blue staining, the portion of the PVDF membrane carrying the proteins of interest was cut out and used. This sample was divided further into two portions, one containing a higher molecular weight protein and one containing a lower molecular weight protein. The N-terminal amino acid sequence of each protein was determined using a gas-phase protein sequencer (476A, Applied Biosystems, Tokyo, Japan) as described in Chapter 1.

cDNA library construction and screening

Total RNA was isolated from 'Erdi Botermo' styles with stigmas at the balloon stage of development as described in Chapter 1. Double-stranded cDNA was synthesized from the poly $(A)^+$ RNA isolated from the total RNA, cloned into Lambda ZAP II vector (Stratagene, La Jolla, Calif.), and packaged in vitro using MaxPlax Packaging Extract Kit (Epicentre Technologies, Madison, Wis.) as described in Chapter 1.

Pru-T2 primer (see Chapter 1) was used in 3' RACE under the PCR condition identical to that used in Chapter 1. The PCR products were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, Wis.). DNA sequences of the inserts of several clones were determined using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Putative 3'RACE clones were labeled by random primer incorporation of DIG-dUTP (Boehringer Mannheim, Tokyo, Japan) and used as probes to screen the primary cDNA library consisting of 1×10^6 plaque forming units. The cDNA library was screened as described in Chapter 1, and selected clones were converted to pBluescript plasmids for DNA sequencing as described above. The deduced amino acid sequences of two kinds of cDNAs from 'Erdi Botermo' and four of cDNAs from 'Rheinische Schattenmorelle' were obtained in this study and these sequences were aligned with sweet cherry S^4-RNase using CLUSTAL X (Thompson et al., 1997).

Genomic DNA blot analysis

Total DNA was isolated from young leaves of 12 sour cherry cultivars and selections except for 'Favorit' by the CTAB method described by Stockinger et al. (1996). Genomic DNA blot analysis was performed as described in Chapter 2. Probe cDNAs were prepared from PCR-amplified fragments of the S^4- and S^6-RNase cDNAs from sweet cherry and radiolabelled with ^{32}P-dCTP as described in Chapter 2.

PCR amplification, cloning, and DNA sequencing of PCR amplified fragments of the S-RNase fragments

As described above for the genomic DNA blot analysis, total DNA was isolated from 11 sour cherry cultivars and selections except for 'Cigany 59' and 'Favorit' and used as template DNA for PCR. PCR procedures were identical to those used by in Chapter 1. PCE-R primer (5'- TGTTT GTTCC ATTCG CYTTC CC -3'), which corresponds to the previously identified C3 conserved domain of rosaceous S-RNases (Ushijima et al., 1998), was designed based on the nucleotide sequences of obtained sour cherry *S*-RNase cDNAs.

After PCR with Pru-C2 and PCE-R primer set, the PCR products were run on 1.5% agarose gel and the DNA bands were visualized by ethidium bromide staining. Furthermore, PCR products from 'Erdi Botermo' were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, Wis.) and their sequences were determined as described above.

3.2.2. Results and Discussion

Evaluation of self-incompatibility or self-compatibility in sour cherry selections

Six cultivars and selections, 'Erdi Nagygyumolcsu', 'Crisana', 'Pandy 38', 'Pandy 114', 'Tschernokorka' and MSU seedling selection I 20 (36), were self-incompatible and no self-pollen tubes reached the ovule 72 h after pollination in any of the 10 pistils observed (Fig. 3.1A), while outcross pollen tubes reached the ovule (Fig. 3.1B). Self-pollen tube growth of these selections was inhibited in the middle part of stylar tissue (Fig. 3.1A), and swelling of pollen tube tips, which is a typical reaction of gametophytic SI, was observed in the self-incompatible sour cherry cultivars and selections (Fig. 3.1C). Seven cultivars and selections, 'Cigany 59', 'Erdi Botermo', 'Montmorency', 'Favorit', 'Rheinische Schattenmorelle', 'Surefire', and 'Ujfehertoi Furtos' were self-compatible because self-pollen tubes successfully reached the ovule (Fig. 3.1D). These results were consistent with previous observations (Iezzoni, 1996; Lansari and Iezzoni, 1990; Redalen, 1984a, b).

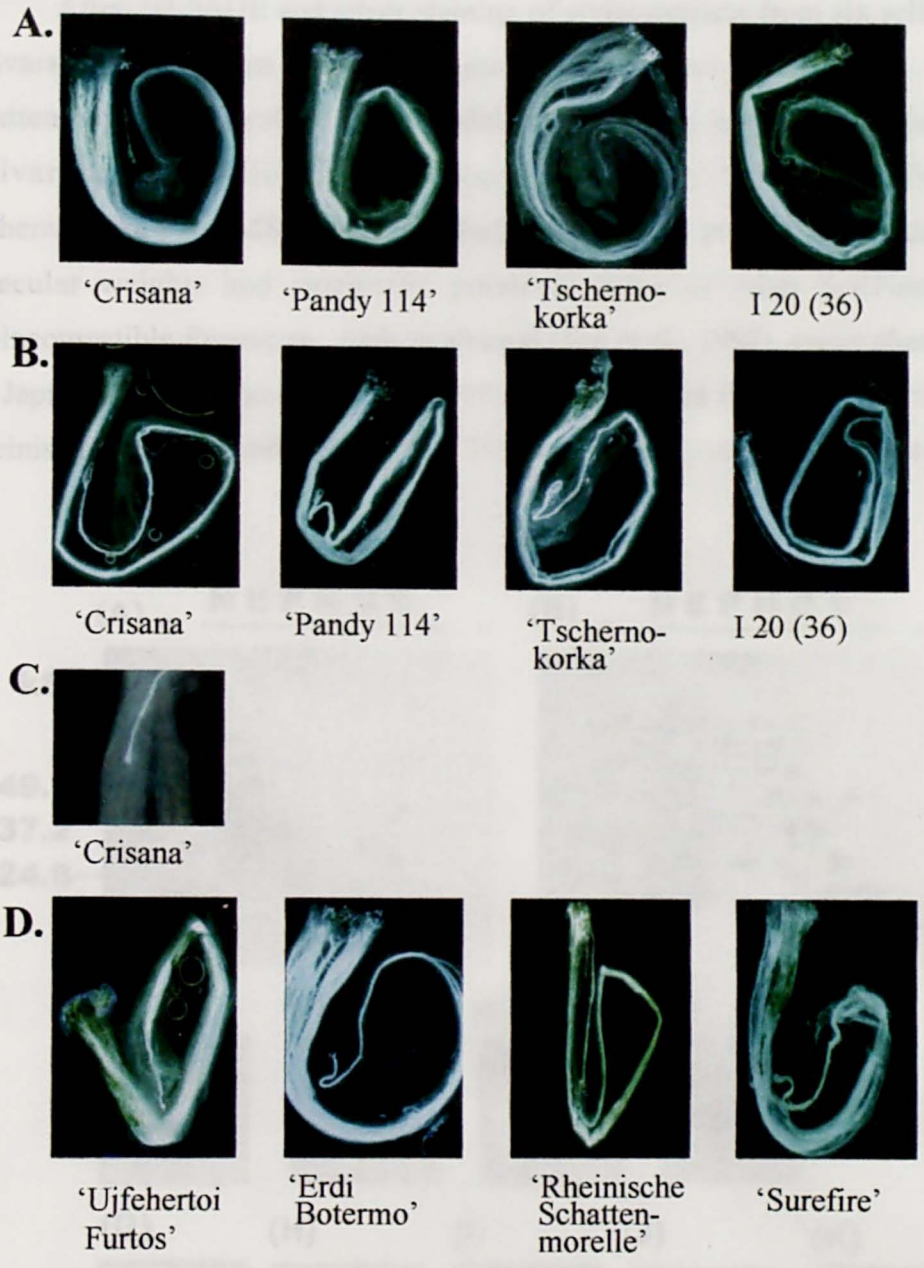

Fig. 3.1. Pollen tube growth in sour cherry pistils. The pistils were purposely curved before the photographs were taken. (A) self-pollinated styles of the self-incompatible cultivars and selections, 'Crisana', 'Pandy 114', 'Tschernokorka', and MSU seedling selection I 20 (36). (B) Out-crossed styles of 'Crisana', 'Pandy 114', 'Tschernokorka', and MSU seedling selection I 20 (36). (C) Swelling of the pollen tube tip in self-pollinated pistil of 'Crisana'. (D) Self-pollinated styles of the self-compatible selections, 'Ujfehertoi Furtos', 'Erdi Botermo', 'Rheinische Schattenmorelle', and 'Surefire'.

Protein assay

After 2D-PAGE and silver staining of stylar extracts from six self-compatible cultivars and selections ('Erdi Botermo', 'Montmorency', 'Favorit', 'Rheinische Schattenmorelle', 'Surefire', and 'Ujfehertoi Furtos') and five self-incompatible cultivars and selections ['Erdi Nagygyumolcsu', 'Crisana', 'Pandy 114', 'Tschernokorka', and MSU seedling selection I 20 (36)], protein spots that had similar molecular weights and isoelectric points to those of other S-RNases in other self-incompatible *Prunus* sp., such as almond (Tao et al., 1997), sweet cherry (Fig. 1.2) and Japanese plum (Yamane et al., 1999), were detected (Fig. 3.2). Furthermore, for 'Rheinische Schattenmorelle' and 'Erdi Botermo', the stylar proteins were shown to be

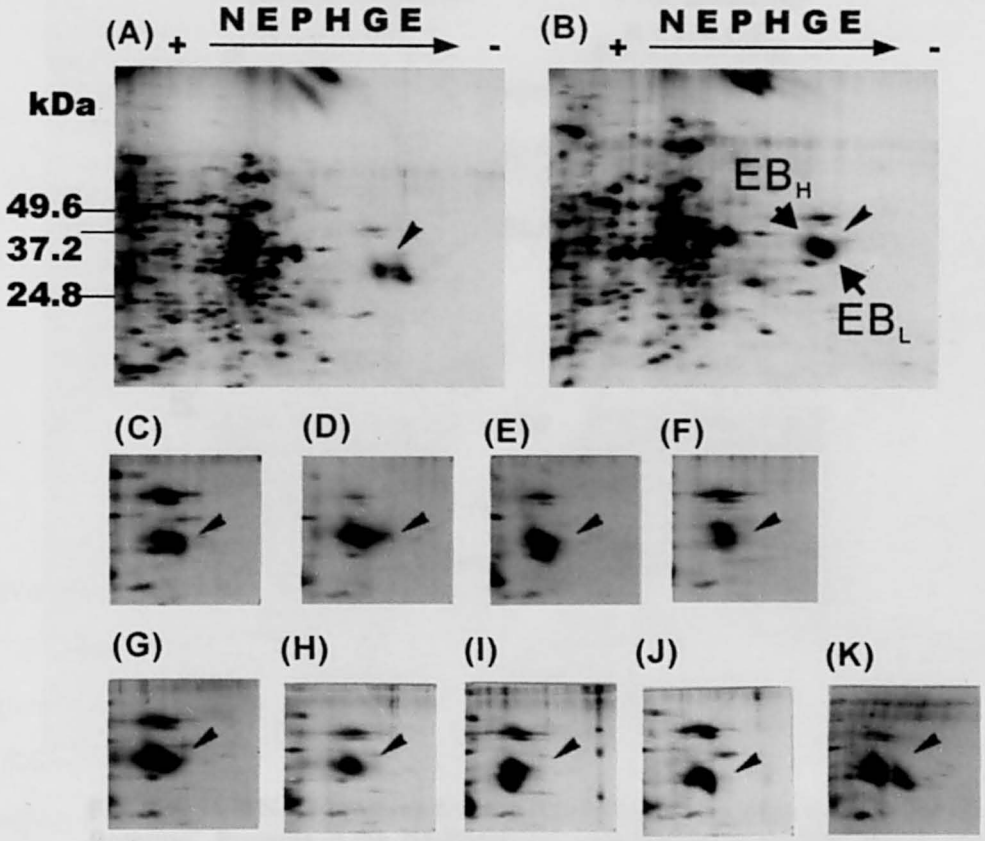

Fig. 3.2. Identification of S-RNases of 11 sour cherry cultivars and selections. (A-F) stylar proteins from six self-compatible cultivars and (G-K) five self-incompatible cultivars and selection were separated by 2D-PAGE and detected by silver staining. S-RNase spots are marked with arrowheads. (A) 'Rheinische Schattenmorelle', (B) 'Erdi Botermo', (C) 'Montmorency', (D) 'Ujfehertoi Furtos', (E) 'Favorit', (F) 'Surefire', (G) 'Pandy 114', (H) 'Crisana', (I) 'Erdi Nagygyumolcsu', (J) 'Tschernokorka', and (K) MSU seedling selection I 20 (36). For 'Erdi Botermo', two kinds of spots (EB_H and EB_L) are partially overlapped (see text).

glycoproteins that contained sugar chains reacting with Con A (Fig. 3.3A) and exhibited an immuno-reaction with the anti pistil S^c-serum raised against S^c-RNase of almond (Fig. 3.3B). This result indicates that they are immunologically similar to *S*-RNases of other *Prunus* spp. The putative *S*-RNase spot from 'Erdi Botermo' was divided into two regions of higher (spot EB_H) and lower (spot EB_L) molecular weight (Fig. 3.2). The N-terminal amino acid sequences of these proteins were homologous to those of S-RNases of other *Prunus* spp. (Fig. 3.4) (Tao et al., 1997; Yamane et al., 1999).

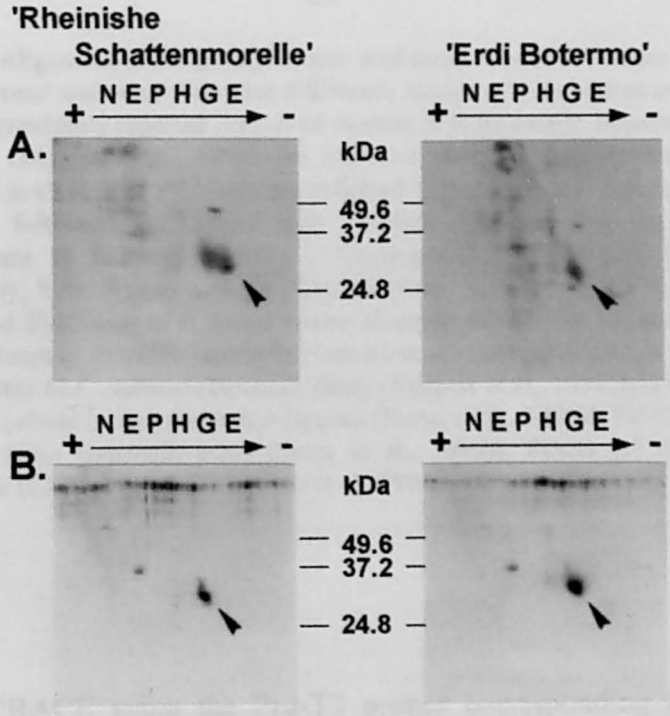

Fig. 3.3. S-RNase proteins from 'Rheinische Schattenmorelle' and 'Erdi Botermo'. Proteins in the 2D-PAGE gel were blotted to a PVDF membrane and (A) detected by glycoprotein staining with Con A and (B) immunodetection with the anti-S^c-serum prepared from almond S^c-RNase. S-RNase spots are marked with *arrowheads*.

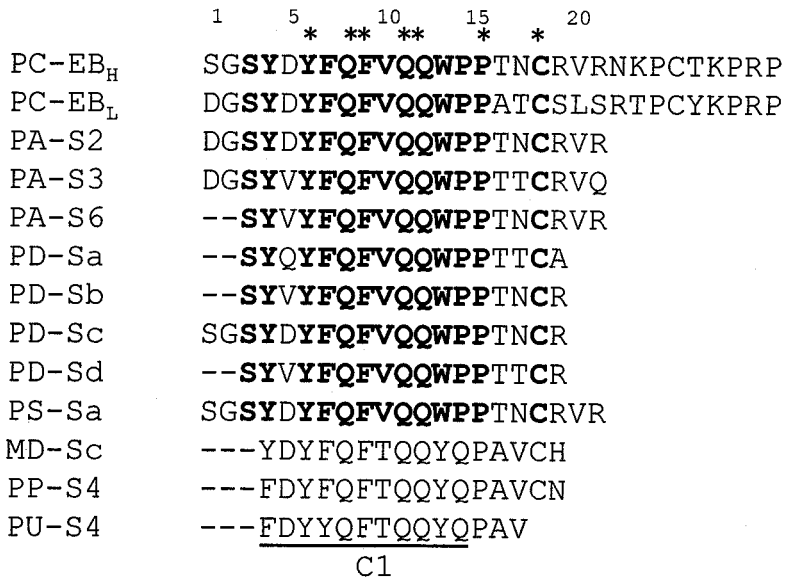

Fig. 3.4. Alignment of N-terminal amino acid sequences of S-RNases of sour cherry 'Erdi Botermo' and other rosaceous *S*-RNases. Amino acid sequences corresponding to the C1, a previously reported conserved domain of S-RNases in Rosaceae (Ishimizu et al., 1998; Ushijima et al., 1998), are indicated at the bottom. Amino acid residues conserved in all *Prunus* S-RNases are indicated by bold face and those conserved in all rosaceous S-RNases are marked with asterisks. Sequence data for the S-RNases included are as follows: PC-EB$_{H,\ L}$ [corresponding to the spot EB$_H$ and EB$_L$, respectively, from *Prunus cerasus* ('Erdi Botermo' sour cherry)]; PA-S2, S3, and S6 [S^2, S^3, and S^6-RNases of *P. avium* (sweet cherry)]; PD-Sa, Sb, Sc, and Sd [S^a, S^b, S^c, and S^d-RNases of *P. dulcis* (almond) (Tamura et al., 2000; Ushijima et al., 1998)]; PS-Sa [S^a-RNase of *P. salicina* (Japanese plum) (Yamane et al., 1999)]; MD-Sc [S^c-RNase of *Malus sylvestris* var. *domestica* (apple) (Sassa et al., 1996)]; PP-S4 [S^4-RNase of *Pyrus pyrifolia* (Japanese pear) (Sassa et al., 1996)]; PU-S4 [S^4-RNase of *Pyrus ussuriensis* (Chinese pear) (Tomimoto et al., 1996)].

cDNA cloning

For 3'RACE using the Pru-T2 primer corresponding to the signal peptide sequence of S-RNases of sweet cherry, 10 clones that could be "nested" PCR-amplified by the AS1 (see Chapter 1) and Pru-C4R primer set as described in Chapter 1, were obtained. Nine clones had the same nucleotide sequence and their deduced amino acid sequence contained the N-terminal amino acid sequence of the spot EB$_H$ from 'Erdi Botermo' (Figs. 3.2, 3.4). The remaining one clone contained the N-terminal amino acid sequence encoding the spot EB$_L$ from 'Erdi Botermo' (Figs. 3.2, 3.4). These two 3'RACE clones were further PCR amplified by Pru-C2 and Pru-C4R primers and used as probes to screen the cDNA library. About 5000 plaques were

screened, and several positive plaques were converted to pBluescript and DNA sequenced. Two different kinds of full-length cDNA clones corresponding to two spots, EB_H and EB_L, were obtained. The coding region of one cDNA clone encoding the spot EB_H was exactly the same as the S^4-RNase cDNA of sweet cherry (Fig. 3.5). The other cDNA clone encoding the spot EB_L contained five conserved regions, seven cysteine residues and an N-glycosylation site conserved among other rosaceous S-RNases (Ushijima et al., 1998) but did not share the same DNA sequence with any of the known S-RNase sequences, suggesting that it encodes a novel S-RNase (named S^a-RNase in this study) in sour cherry (Fig. 3.5).

Fig. 3.5. Amino acid sequence alignment of two S-RNases, EB_L and EB_H, from 'Erdi Botermo' sour cherry and sweet cherry S^4-RNase. The alignment was generated by CLUSTAL X (Thompson et al., 1997). Gaps are marked by dashes. The five conserved regions, C1, C2, C3, RC4, and C5 (Ushijima et al., 1998) are shown and boxed, and hypervariable region, RHV (Ushijima et al., 1998), reported in rosaceous S-RNases are shown under the alignment and underlined. Sequence data for the *S*-RNases included are as follows: PC-EB_L, EB_H [corresponding to the spot EB_L and EB_H, respectively, from *Prunus cerasus* ('Erdi Botermo' sour cherry)], PA-S4 [S^4-RNase of *P. avium* (sweet cherry)]. PC-EB_H and PA-S4 sequences are shown in bold face because they are exactly the same.

3' RACE cDNA clones encoding four different S-RNases were cloned and sequenced from 'Rheinische Schattenmorelle'. The partial amino acid sequence of one of them was exactly the same as the S^6-RNase of sweet cherry (Fig. 3.6). The partial amino acid sequence of the S^a-RNase from 'RS' was identical to the sequence of the S^a-RNase from 'EB' (Fig. 3.6). The deduced amino acid sequences from the remaining two cDNAs contained the two active domains shared by other T2/*S* type RNases and the five regions that are conserved among rosaceous S-RNases (Ushijima et al., 1998). However, their sequences were not identical to the DNA sequences of any of the known S-RNases, suggesting that they encode novel S-RNases (named S^b- and S^c-RNases in this study). The novel S^a-, S^b- and S^c-RNases share 63 to 80 % amino acid sequence identity with other sweet cherry S-RNases, which is within the range of amino acid sequence identity observed among *Prunus* S-RNases.

S-RNase alleles in twelve sour cherry cultivars and selections

Hybridization signals with the cDNA probes encoding S^4-, and S^6-RNases of sweet cherry were observed with all 12 sour cherry cultivars and selections tested (Fig. 3.7). With *Hin*dIII or *Xba*I digestions, four different *S*-haplotypes seemed to be present in three selections, 'Cigany 59', 'Erdi Nagygyumolcsu', and 'Rheinische Schattenmorelle', and three different *S*-haplotypes were present in all other selections (Table 3.2; Fig. 3.7). Three selections, 'Crisana', 'Pandy 38', and 'Pandy 114', yielded the same banding pattern for both blots, which is consistent with the fact that 'Crisana' and 'Pandy' represent selections from the same landrace (Iezzoni, 1996).

Based on the RFLP analysis, 'Erdi Botermo' appears to have three different *S*-haplotypes: S^4, S^a, and S^6 (Table 3.2; Fig. 3.7). In the *Xba*I digest, 'Erdi Botermo' exhibited the fragment that corresponds to the sweet cherry S^6-haplotype, however, the fragment identified from the *Hin*dIII digest showed a different size from that of the sweet cherry S^6-haplotype. To resolve this discrepancy, the 'Erdi Botermo' fragment that was associated with the S^6-RNase gene was amplified by PCR (Fig. 3.8) and sequenced. Sequencing data showed this fragment contained a partial DNA sequence unique to the S^6-RNase gene (data not presented). Therefore, we propose that 'Erdi Botermo' has a S^6-RNase gene which has an altered *Hin*dIII cut site. The mutation might affect transcription of the allele as cDNA encoding S^6-RNase could not be

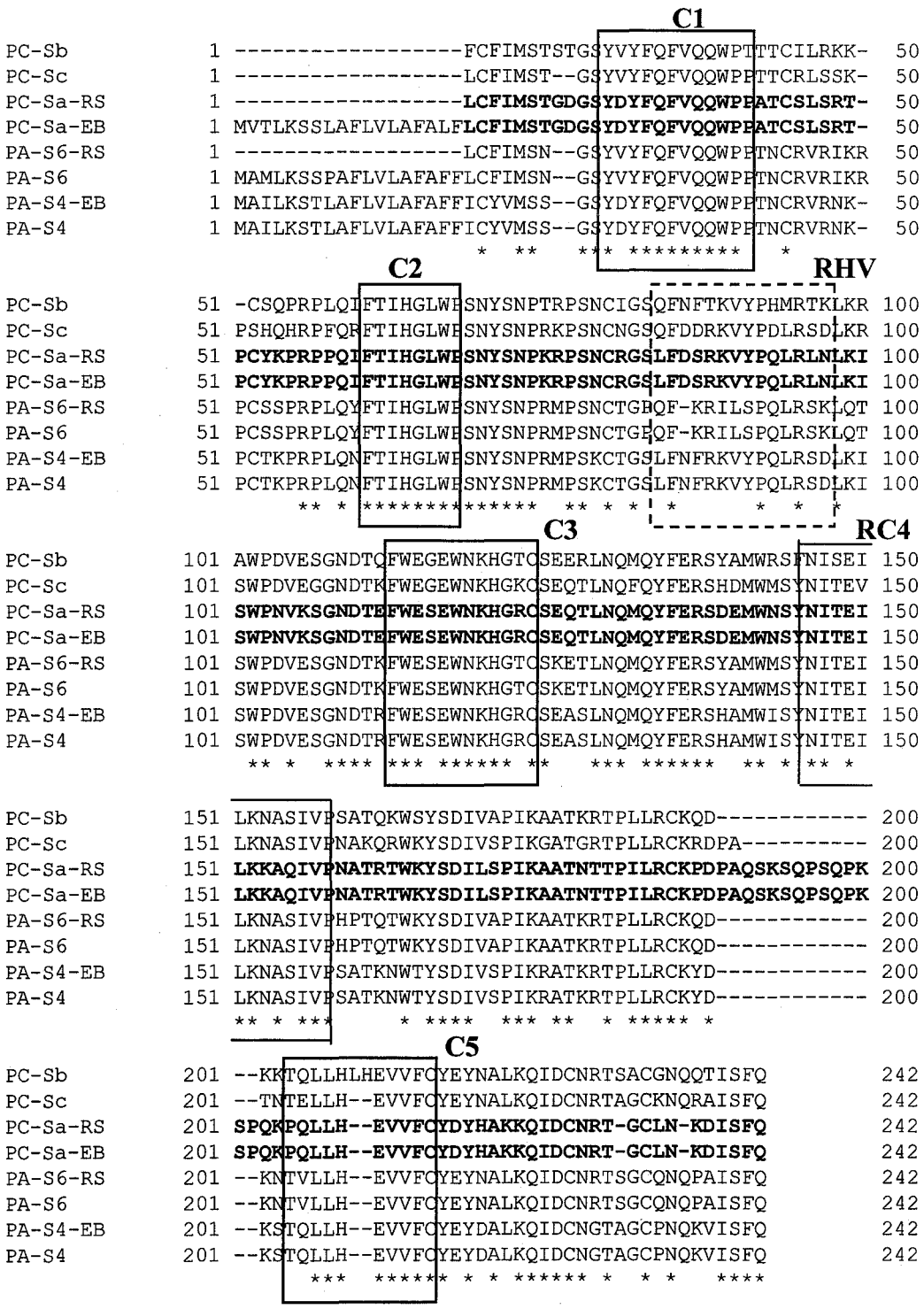

Fig. 3.6. Amino acid sequence alignment of four S-RNases from 'Rheinische Schattenmorelle (RS)', S^a, S^b, S^c and S^6, two from 'Erdi Botermo (EB)', S^a and S^4, and the sweet cherry S^4- and S^6-RNases. The alignment was generated by CLUSTAL X (Thompson et al. 1997). Gaps are marked by dashes. The five conserved regions, C1, C2, C3, RC4 and C5 (Ushijima et al. 1998) are marked with solid boxes, and the hypervariable region, RHV (Ushijima et al. 1998), reported in rosaceous S-RNases is marked with a dashed box. Amino acid residues conserved in between Sa-RNases from RS and EB are indicated by bold face and those conserved in all S-RNases are marked with asterisks under the sequences.

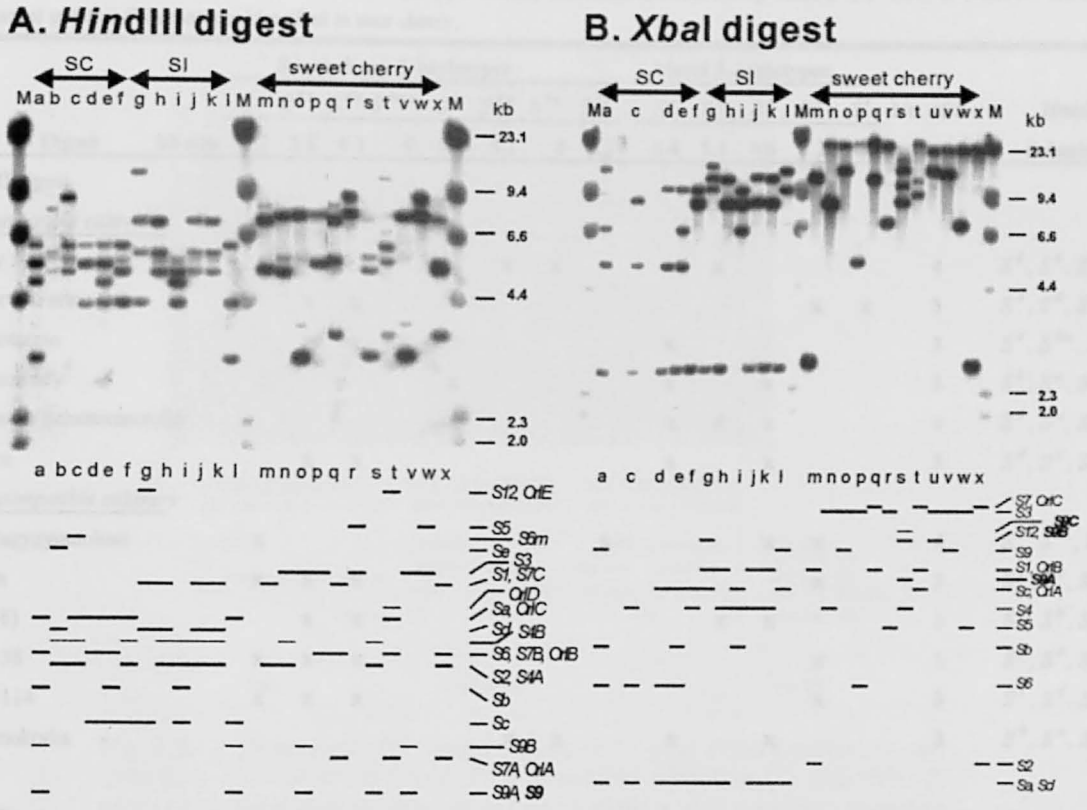

Fig. 3.7. Genomic DNA blot analysis of 12 sour cherry selections. Six micrograms of genomic DNA were digested by (A) *Hin*dIII or (B) *Xba*I and hybridized to the cDNAs encoding (A) S^6-RNase or (B) S^4-RNase, respectively. M: Lambda/*Hin*dIII marker. (a) 'Cigany 59', (b) 'Ujfehertoi Furtos', (c) 'Erdi Botermo', (d) 'Montmorency', (e) 'Rheinische Schattenmorelle', (f) 'Surefire', (g) 'Erdi Nagygyumolcsu', (h) 'Crisana', (i) MSU seedling selection I 20 (36), (j) 'Pandy 38', (k) 'Pandy 114', (l) 'Tschernokorka', (m) 'Early Rivers' (S^1S^2), (n) 'Bing' (S^3S^4), (o) 'Burlat' (S^3S^9), (p) 'Gold' (S^3S^6), (q) 'Charger' (S^1S^7), (r) 'Goucher' (S^3S^5), (s) 'Inge' (S^4S^9), (t) 'Orleans 171' ($S^{10}S^{11}$), (u) 'Schneiders' (S^3S^{12}), (v) 'Mona' (S^3S^9), (w) 'Hedelfingen' (S^3S^5), and (x) 'Guigne d'Annonay' (S^2S^7). *Orl* represents S^{10} and S^{11} found in 'Orleans 171'. If more than one fragment corresponds to an allele, A is used to designate the smallest fragment, B the next smallest, etc. The fragments corresponding to the S^9-haplotype from 'Inge' are bold, whereas the fragments corresponding to the S^9-allele from 'Burlat' are not bold.

obtained by screening of the stylar cDNA library or 3'RACE from the mRNA from the style of 'Erdi Botermo'. This conclusion is also supported by the fact that the protein spot corresponding to S^6-RNase was absent in the 2D-PAGE profile of 'Erdi Botermo' (Fig. 3.2B).

Table 3.2. Putative S-haplotypes of 12 sour cherry selections predicted by their RFLP kb size on Southern blots following *Hin* dIII and *Xba* I digest. S^1, S^4, S^6, S^9, and S^{12} refer to S-haplotypes already identified in sweet cherry while S^a, S^b, S^c, S^d, and S^e are assigned to the novel putative S-haplotypes identified in sour cherry.

	Sweet cherry S-haplotypes								Novel S-haplotypes						
	S^1	S^{4z}	S^{4z}	S^{6my}	S^6	S^{9x}	S^{9x}	S^{12}	S^a	S^b	S^c	S^d	S^e	No. of S-	Identified
Digest kb size	8.7	5.6	6.1	9	5.8	3.1	4	12	6.4	5.1	4.6	6.2	9.6	haplotypes	S-haplotypes
Hin dIII digest															
Self-compatible cultivars															
Cigany 59					x	x	x		x	x				4	S^6, S^9, S^a, S^b
Ujfehertoi Furtos		x	x									x	x	3	S^4, S^d, S^e
Erdi Botermo		x	x	x					x					3	S^4, S^{6m}, S^a
Montmorency					x				x		x			3	S^6, S^a, S^c
Rheinische Schattenmorelle					x				x	x	x			4	S^6, S^a, S^b, S^c
Surefire		x	x						x		x			3	S^4, S^a, S^c
Self-incompatible cultivars															
Erdi Nagygyumolcsu	x							x			x	x		4	S^1, S^{12}, S^c, S^d
Crisana	x	x	x									x		3	S^1, S^4, S^d
I 20 (36)		x	x							x	x			3	S^4, S^b, S^c
Pandy 38	x	x	x									x		3	S^1, S^4, S^d
Pandy 114	x	x	x									x		3	S^1, S^4, S^d
Tschernokorka						x	x		x		x			3	S^9, S^a, S^c

Xba I digest

	Sweet cherry S-haplotypes					Novel S-haplotypes						
	S^1	S^4	S^6	S^9	S^{12}	S^a, S^d		S^b	S^c		No. of S-	Identified
kb size	13	8.8	5.5	15	16		2.4	5	9.4		haplotypes	S-haplotypes
Self-compatible cultivars												
Cigany 59			x	x			x	x			4	S^6, S^9, S^a, S^b
Erdi Botermo		x	x				x				3	S^4, S^6, S^a
Montmorency			x				x		x		3	S^6, S^a, S^c
Rheinische Schattenmorelle			x				x	x	x		4	S^6, S^a, S^b, S^c
Surefire		x					x		x		3	S^4, S^a, S^c
Self-incompatible cultivars												
Erdi Nagygyumolcsu	x				x		x		x		4	S^1, S^{12}, S^c, S^d
Crisana	x	x					x				3	S^1, S^4, S^d
I 20 (36)		x						x	x		3	S^4, S^b, S^c
Pandy 38	x	x					x				3	S^1, S^4, S^d
Pandy 114	x	x					x				3	S^1, S^4, S^d
Tschernokorka				x			x		x		3	S^9, S^a, S^c

[z] The S^4-haplotype band is identified by two fragments of unequal intensity. The 6.1 kb band is faint and sometimes not found because of short exposure time as described in chapter 2.

[y] 'Erdi Botermo' has a mutated S^{6m}-haplotype which has an altered *Hin* dIII cut site (see text).

[x] The S^9-haplotype band is identified by two fragments of unequal intensity. The 4 kb band is faint and sometimes not found because of short exposure time as described in chapter 2.

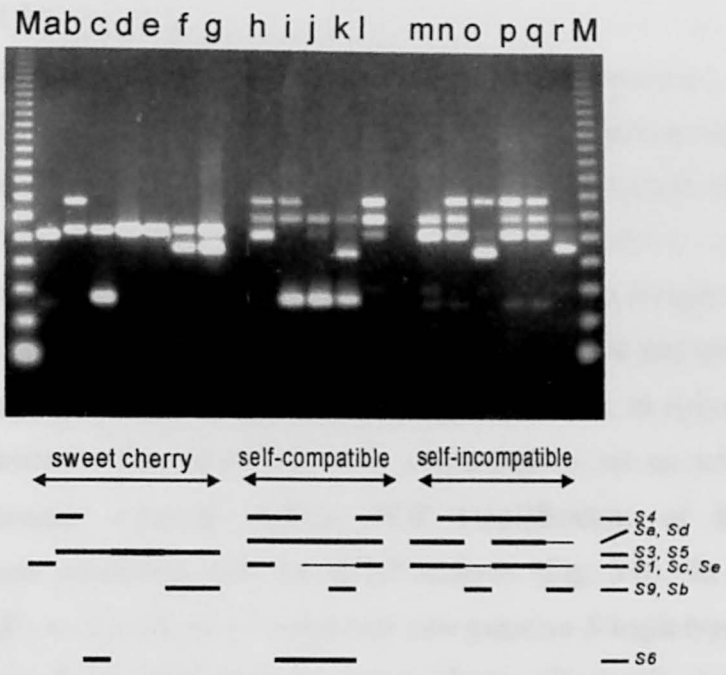

Fig. 3.8. PCR analysis for *S*-haplotypes of 11 sour cherry cultivars and selection. Genomic DNA was PCR amplifed with Pru-C2 and PCE-R primer set, separated on agarose gel and detected with ethidium bromide staining. M: 123 bp DNA ladder. (a) 'Early Rivers' (S^1S^2), (b) 'Bing' (S^3S^4), (c) 'Gold' (S^3S^6), (d) 'Hedelfingen' (S^3S^5), (e) 'Goucher' (S^3S^5), (f) 'Burlat' (S^3S^9), (g) 'Mona' (S^3S^9), (h) 'Ujfehertoi Furtos', (i) 'Erdi Botermo', (j) 'Montmorency', (k) 'Rheinische Schattenmorelle', (l) 'Surefire', (m) 'Erdi Nagygyumolcsu', (n) 'Crisana', (o) MSU seedling selection I20 (36), (p) 'Pandy 38', (q) 'Pandy 114', and (r) 'Tschernokorka'.

The band corresponding to the S^1-haplotype of sweet cherry was found in four SI cultivars and selections, 'Erdi Nagygyumolcsu', 'Crisana', 'Pandy 38', and 'Pandy 114'. The band corresponding to the S^4-haplotype was found in three self-compatible cultivars ('Erdi Botermo', 'Surefire', and 'Ujfehertoi Furtos') and in four self-incompatible cultivars and selection ['Crisana', 'Pandy 38', 'Pandy 114', and MSU seedling selection I 20 (36)] (Table 3.2; Fig. 3.7). Similarly, four self-compatible selections ('Cigany 59', 'Erdi Botermo', 'Montmorency', and 'Rheinische Schattenmorelle') were shown to have the S^6-haplotype of sweet cherry. One self-compatible cultivar ('Cigany 59') and one self-incompatible cultivar ('Tschernokorka') were shown to have the S^9-haplotype of the sweet cherry cultivars 'Burlat' and 'Mona' (Tables 3.1 and 3.2, Fig. 3.7). 'Erdi Nagygyumolcsu' exhibited the

band corresponding to the S^{12}-haplotype of the sweet cherry cultivar 'Schneiders' (Tables 3.1 and 3.2, Fig. 3.7).

Five self-compatible cultivars ('Cigany 59', 'Erdi Botermo', 'Montmorency', 'Rheinische Schattenmorelle', and 'Surefire') and one self-incompatible cultivar ('Tschernokorka') yielded the band that was considered to encode the novel putative S-RNase from 'Erdi Botermo' and 'Rheinische Schattenmorelle', named S^a-RNase in this study (Table 3.2, Fig. 3.7). The other four novel putative *S*-haplotypes, named S^b, S^c, S^d, and S^e of sour cherry, were observed in several cultivars and selection (Table 3.2, Fig. 3.7). These putative *S*-haplotypes were assigned a letter, as opposed to numerical designations, because they have yet to be confirmed to act as self-incompatibility haplotypes through crossing studies. PCR amplification of the sour cherry *S*-haplotypes was consistent with the RFLP analysis (Fig. 3.8). To date, at least 14 *S*-haplotypes, S^1- to S^7-, S^9- to S^{13}-, and two new putative *S*-haplotypes, both found in 'NY1625', have been confirmed in sweet cherry (Boskovic and Tobutt, 2001; Sonneveld et al., 2001; Wiersma et al., 2001, see Chapter 2). Although the five novel *S*-haplotypes, S^a- to S^e-, identified in this study were different from the sweet cherry S^1- to S^7-, S^9- to S^{12}-haplotypes, it is possible that these novel *S*-haplotypes are present in other sweet cherry clones.

Pollen tube growth studies of sour cherry and sweet cherry interspecific crosses

As described above, the pistil *S*-alleles that 'Crisana' and 'Tschernokorka' possesses were shown to be S^1-, S^4-, and S^d-alleles and S^9-, S^a-, and S^c-alleles, respectively (Table 3.3). To demonstrate whether the recognition of common *S*-haplotypes has been maintained in spite of polyploidization in sour cherry, inter-specific crosses were done.

'Satonishiki' (S^3S^6) pollen was able to grow the full length of both 'Crisana' ($S^1S^4S^d$) and 'Tschernokorka' ($S^9S^aS^c$) styles (Table 3.3). 'Rainier' (S^1S^4) pollen was able to grow the full length of the 'Tschernokorka' styles (Table 3.3). However, 'Rainier' pollen tube growth was arrested half way down the 'Crisana' styles (Table 3.3) and swelling was observed at the pollen tube tips. This suggests that the S^1- and S^4-RNases of 'Crisana' were able to recognize S^1- and S^4-pollen of 'Rainier' and inhibited their pollen tube growth.

'Tschernokorka' pollen grew the full length of both 'Rainier' and 'Schmidt' (S^2S^4) styles. (Table 3.4). This was expected since 'Tschernokorka' has no *S*-haplotypes in common with either of the sweet cherry cultivars. 'Crisana' pollen was also able to grow the full length of 'Schmidt' styles; however, 'Crisana' pollen was arrested halfway down the 'Rainier' styles (Table 3.4). Six types of pollen from 'Crisana' would be possible: heteroallelic (i.e. S^1S^4, S^1S^d, S^4S^d) and homoallelic (S^1S^1, S^4S^4, S^dS^d) depending on which allele is in double dose. Because all the pollen tube growth were inhibited in the style containing S^1- and S^4-RNases, S^1- and S^4-RNases from 'Rainier' were able to recognize and degrade the 2x pollen tubes from 'Crisana' that would either be heteroallelic for the S^1, S^4 and S^d pollen *S*-alleles or homoallelic for the S^1 or S^4 pollen *S*-alleles. Hence, this suggests that a match between any stylar S-RNase and either of the two pollen *S*-alleles in the 2x pollen causes an incompatible interaction. This is contrary to the hypothesis that pollen containing two different pollen *S*-alleles loses its SI phenotype due to a competition between the pollen *S*-alleles, thus any pollen containing two different pollen *S*-alleles is compatible with any plant, regardless of its *S*-haplotype combinations suggested by Golz et al., (1999, 2001).

Table 3.3. Cross-(in)compatibility results for pollination of sour cherry styles with sweet cherry pollen based on examination of pollen tube growth in styles 72 hours after pollination

Style parent	Pollen parent (*S*-haplotype)	
(S-RNases)[a]	'Rainier' (S^1S^4)	'Satonishiki' (S^3S^6)
'Crisana' ($S^1S^4S^d$)	Incompatible	Compatible
'Tschernokorka' ($S^9S^aS^c$)	Compatible	Compatible

[a] Three different *S*-RNases have been identified in each parent. At this time, it is not known which of the three *S*-RNases is present in two copies.

Table 3.4. Cross-(in)compatibility results for pollination of sweet cherry styles with sour cherry pollen based on examination of pollen tube growth in styles 72 hours after pollination

Style parent	Pollen parent (S-RNases) [a]	
(*S*-haplotype)	'Crisana' ($S^1S^4S^d$)	'Tschernokorka' ($S^9S^aS^c$)
'Ranier' (S^1S^4)	Incompatible	Compatible
'Schmidt' (S^2S^4)	Compatible	Compatible

[a] Three different *S*-RNases have been identified in each parent. At this time, it is not known which of the three *S*-RNases is present in two copies.

Possible mechanism controlling self-incompatibility and self-compatibility in sour cherry

Lewis (1947) proposed that heterogenic pollen loses its self-incompatibility phenotype, which causes breakdown of self-incompatibility in polyploid plants with diploid relatives possessing a gametophytic self-incompatibility system. To date, some observations that are consistent with this hypothesis have been obtained. As demonstrated in a tissue culture-derived *Lycopersicon peruvianum* Mill. selection, when an $S^1S^1S^2S^2$ individual was self-pollinated, only S^1S^2 pollen achieved fertilization (Chawla et al., 1997). In artificially induced tetraploid lines of *Petunia* × *hybrida* Hort. Vilm.-Andr., the obtained $S^{B1}S^{B1}S^{B1}S^{B1}$ homoallelic tetraploid remained self-incompatible, whereas the $S^{B1}S^{B1}S^{B2}S^{B2}$ heteroallelic tetraploid became self-compatible (Entani et al., 1999). In addition, Golz et al. (1999, 2001) found breakdown of self-incompatibility in mutated diploid plants with an extra *S*-haplotype generated by irradiation. The hypothesis of Lewis (1947) and the observations of Chawla et al. (1997), Entani et al. (1999) and Golz et al., (1999, 2001) indicate that tetraploid plants with more than two *S*-haplotypes are self-compatible, and self-incompatibility is found only when all of the four *S*-haplotypes are the same. In this study, however, we identified three or four different *S*-haplotypes in each self-incompatible sour cherry selection. If these *S*-alleles are functional in both pollen and stylar parts, it appears that our results are inconsistent with the hypothesis of Lewis (1947). It is possible that self-incompatibility and self-compatibility in sour cherry is controlled by not only *S*-genes but also other factors, such as the HT-protein of *Nicotiana* sp. (McClure et al., 1999). Alternatively, it is possible that the identified *S*-haplotypes could be mutated such that *S*-allelic products of pollen part and/or stylar part are nonfunctional. The genetic control of self-incompatibility and self-compatibility in naturally occurring tetraploid sour cherry is apparently more complicated than that in artificially produced tetraploid tomato (*Lycopersicon peruvianum*) (Chawla et al., 1997).

3.3. The inheritance of *S*-haplotypes and the segregation of self-incompatibility and self-compatibility phenotypes in 80 F_1 progeny of sour cherry

In sour cherry, it was known that self-incompatible selections can result from crosses between two self-compatible parents (Lansari and Iezzoni, 1990). For example, a sour cherry linkage mapping population generated by crossing two self-compatible sour cherry cultivars, 'Rheinische Schattenmorelle' × 'Erdi Botermo', segregates for self-incompatibility and self-compatibility (Lansari and Iezzoni, 1990; Wang et al., 1998). The previous section demonstrated the existence of the S-RNases that are associated with gametophytic self-incompatibility in *Prunus*, in the styles of both self-incompatible and self-compatible cultivars and selections of sour cherry. Furthermore, it was suggested that heteroallelic pollen alone may be insufficient to cause self-compatibility in tetraploid sour cherry, in contrast to the other reported polyploid plants whose self-compatibility is gained only by heteroallelic pollen (Chawla et al., 1997; Entani et al., 1999; Golz et al., 1999, 2001). To further investigate the genetic mechanism of self-incompatibility and self-compatibility in sour cherry, the cross between 'Rheinische Schattenmorelle' and 'Erdi Botermo', in which the progenies are segregated for self-incompatibility and self-compatibility was used in this study. The 'Rheinische Schattenmorelle' and 'Erdi Botermo' mapping parents had the pistil *S*-alleles, $S^aS^bS^cS^6$ and $S^aS^4S^{6m}$, respectively, as described in the previous section. The inheritance and linkage map locations of the pistil *S*-alleles from 'Rheinische Schattenmorelle' and 'Erdi Botermo' were compared with information from other *Prunus* species. Furthermore, the segregation of self-incompatibility and self-compatibility phenotype were investigated. Then, the hypothesis for the genetic control of self-incompatibility and self-compatibility in tetraploid sour cherry is proposed based on the inheritance of *S*-haplotypes and self-incompatibility phenotypes in the progeny.

3.3.1. Materials and Methods

Plant material

A pseudo-testcross mapping population consisting of 80 progeny from the cross between two sour cherry cultivars, 'Rheinische Schattenmorelle' and 'Erdi Botermo' (Wang et al., 1998) maintained at Clarksville Horticultural Experimental Station (Michigan, USA) were used in this study.

DNA isolation and Southern blot analyses from the parents and progeny in the linkage mapping population

Young, unfolded leaves were collected from the parents and the progeny, placed on dry ice, stored at -80 °C overnight and then lyophilized for 48 h. DNA isolation was done using the CTAB method described by Stockinger et al. (1996). 'Erdi Botermo', 'Rheinische Schattenmorelle', and the linkage mapping progeny were evaluated using Southern blotting following *Hin*dIII digest which had been demonstrated to differentiate all the 'Erdi Botermo' and 'Rheinische Schattenmorelle' S-RNases as indicated in the previous section. Genomic DNA blot analysis was performed as described in Chapter 2. Probe cDNAs were prepared from PCR-amplified fragments of the S^4- and S^6-RNase cDNAs from sweet cherry and radiolabelled with ^{32}P-dCTP as described in Chapter 2.

Inheritance and linkage analysis

Segregation of the *S*-haplotypes that were present in one parent but absent in another parent, was tested for their fit to the expected 1:1 (presence:absence) ratio. S^a present in both parents was tested for its fit to a 3:1 (presence:absence) ratio. The most informative markers for linkage mapping from a pseudo-testcross mapping population are single dose restriction fragments (SDRF) that differ between both parents and segregate 1:1 (presence : absence) (Wu et al. 1992). Therefore, *S*-haplotypes which differed between both parents and fit a 1:1 ratio at the 5% level were combined with the existing marker segregation data previously used to construct the 'Rheinische Schattenmorelle' × 'Erdi Botermo' linkage map (Wang et al. 1998).

Linkage analysis was done with JoinMap V2.0 (Stam 1993) using a minimum LOD score of 3.0. Distances are presented in centi-Morgans calculated by the Kosambi function.

Analysis of self-incompatibility trait by observing the self-pollen tube growth in the pistil

To determine whether the 77 flowering individuals of the 'Rheinische Schattenmorelle' x 'Erdi Botermo' mapping population progeny were self-incompatibility or self-compatibility, pollination tests were performed as described in the previous section. Pistils from each of the progeny were pollinated with either self-pollen or with pollen from a collection of several unrelated sour cherry cultivars (out-cross pollen).

3.3.2. Results and Discussion

Inheritance of the S-RNases in the 'Rheinische Schattenmorelle' × 'Erdi Botermo' mapping population

'Rheinische Schattenmorelle' exhibited four fragments of 6.4 kb, 5.8 kb, 5.1 kb, and 4.6 kb which corresponded to the *S*-RNases, S^a, S^6, S^b and S^c, respectively (Fig. 3.9). The four fragments exhibited by 'Erdi Botermo' corresponded to the S-RNases S^{6m} (9 kb), S^a (6.4 kb) and S^4 (6.1 kb and 5.6 kb) (Fig. 3.9).

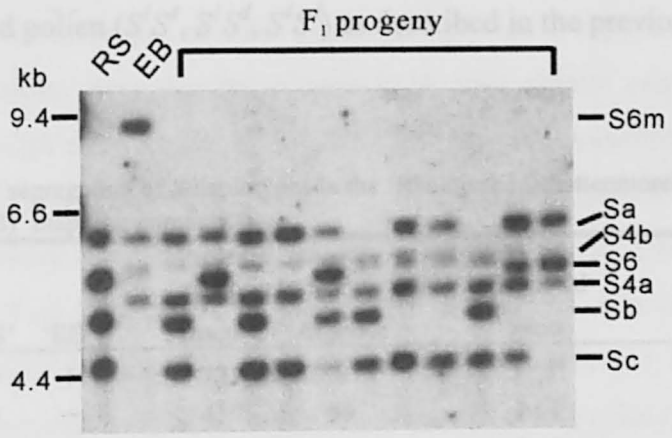

Fig. 3.9. Genomic DNA blot analysis of 'Rheinische Schattenmorelle (RS)', 'Erdi Botermo (EB)' and eleven progeny. Six micrograms of genomic DNA were digested by *Hin*dIII and hybridized to the cDNA encoding the S^6-RNase.

S-RNase segregation in 80 progeny from the 'Rheinische Schattenmorelle' × 'Erdi Botermo' mapping population was determined (Table 3.5). Segregation of the

S-haplotypes that were present in 'Rheinische Schattenmorelle' but absent in 'Erdi Botermo' (S^b, S^c, and S^6) fit the expected 1:1 ratio (Table 3.5, Fig. 3.9). Segregation of the S^a-haplotype which was present in both 'Rheinische Schattenmorelle' and 'Erdi Botermo' was almost a 3:1 ratio. This ratio was expected only if the S^a-haplotype was non-functional for the self-pollen rejection. Segregation for the S^4-haplotype which was present in 'Erdi Botermo' and absent in 'Rheinische Schattenmorelle' did not fit a 1:1 ratio which would be expected if the S^4-haplotype was only present in a single dose. However, segregation of S^4 in 'Erdi Botermo' fit a 5:1 ratio suggesting that there are two S^4-haplotypes exhibiting tetrasomic inheritance and therefore the 'Erdi Botermo' *S*-haplotype is presumed to be $S^{6m}S^4S^4S^a$. The S^{6m} in 'Erdi Botermo' that could be distinguished from the S^6-haplotype in 'Rheinische Schattenmorelle' by RFLP analysis following *Hin*dIII digest (Fig. 3.9), was not present in any of the progeny. This result suggests that only one *S*-allele match between the style and pollen is necessary to render diploid pollen incompatible. Since 'EB' has one S^{6m}-haplotype, all the S^{6m} pollen are expected to be heteroallelic; yet no progeny individual contained the 'EB'-derived S^{6m}-haplotype. This finding is consistent with the inhibition of pollen tube growth of 'Crisana' ($S^1S^4S^d$) in the style of 'Rainier' (S^1S^4) (Table 3.4) since this incompatibility also seemed to be caused by one *S*-allele (S^1, S^4) match between the style (S^1, S^4) and pollen (S^1S^4, S^1S^d, S^4S^d) as described in the previous section.

Table 3.5. RFLP segregation of *S*-haplotypes in the 'Rheinische Schattenmorelle (RS)'x 'Erdi Botermo (EB)' mapping population

			Presence : absence ratio in progeny		Expected		
S-haplotype	'RS'	'EB'	Presence	Absence	ratio	χ^2 value	*p*
S^4	−	+	72	8	5 : 1[z]	2.56	0.110
S^6	+	−	41	39	1 : 1	0.05	0.823
S^{6m}	−	+	0	80	0 : 1		
S^a	+	+	65	15	3 : 1	1.667	0.197
S^b	+	−	32	48	1 : 1	3.200	0.070
S^c	+	−	46	34	1 : 1	1.800	0.180

[z] 5:1 is the expected segregation ratio for an allele that is present in two copies which exhibits tetrasomic inheritance

Linkage analysis of the S-RNases in the 'Rheinische Schattenmorelle' × 'Erdi Botermo' mapping population

The three pistil *S*-allele that fit a 1:1 segregation ratio, S^b, S^c and S^6 (all from the 'Rheinische Schattenmorelle' parent), were used for linkage analysis. The pistil S^b-allele mapped to 'Rheinische Schattenmorelle' linkage group 6 of the framework map constructed by Wang et al. (1998) (Fig. 3.10). The 'Rheinische Schattenmorelle' linkage group 6 consisted of 14 markers spanning 34.4 cM and the S^b-locus mapped 4.5 cM from the marker placed at one end of this linkage group. The other two *S*-locus, S^6 and S^c, were linked to each other at a distance of 23.2 cM and unlinked to any other previously identified markers.

Since sour cherry is an allotetraploid, it is likely that the two *S*-loci would map to homologous linkage groups. The S^b-RNase mapped to the expected position on *Prunus* linkage group 6 indicating that this linkage relationship is maintained among sour cherry, sweet cherry and almond (Ballester et al., 1998). The two other *S*-locus, S^c and S^6, mapped together on what might represent a new homoeologous linkage group 6 that had previously been undetected by Wang et al. (1998) due to low marker density on the sour cherry map. However, S^c and S^6 would have been expected to map to the same location and it is not clear why they are separated by over 20 cM. Selection for or against certain *S*-haplotypes or other alleles in the *S*-locus region could have occurred. In addition meiotic irregularities prevalent in sour cherry can complicate linkage analysis. Although sour cherry is an allotetraploid predominately exhibiting disomic inheritance, it also exhibits tetrasomic inheritance (Beaver et al. 1993) and quadrivalent pairing characteristic of an autotetraploid (Wang 1998). For example, all twenty of the 'Erdi Botermo' metaphase I pollen mother cells (PMC) examined had some non-bivalent pairing with at least one quadrivalent per PMC. Therefore the tetrasomic inheritance exhibited by the 'Erdi Botermo' S^4-haplotype is not unexpected. However, it is more likely that the S^c - S^6 linkage result is caused by an actual change in the physical location of the *S*-locus. This is not unprecedented since a translocation has been confirmed in almond between *Prunus* linkage groups 4 and 6 (P. Arus, unpublished data).

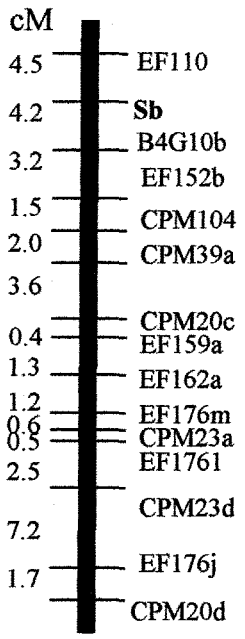

Fig. 3.10. Genetic map for the sour cherry linkage group 6 obtained with the 'Rheinische Schattenmorelle' x 'Erdi Botermo' mapping population showing the location of the S^b-RNase (S^b). The framework map was created by Wang et al. (1998). Markers shown on the right are identified by the probe followed by a letter (i.e., *a*, *b*, *c*, etc.) when more than one marker is generated from a single probe.

Determination of self-incompatibility phenotypes and possible genetic control of self-incompatibility and self-compatibility in the 'RS' x 'EB' mapping populations

Pollen tube growth tests showed that both self- and outcross-pollen grew the full length of 'Rheinische Schattenmorelle' and 'Erdi Botermo' styles, which confirms that both cultivars are self-compatible. The self-incompatibility phenotype could be determined for 59 of the 80 individuals, that represent six of 15 different RFLP types observed in the 80 progeny (Table 3.6). The self-incompatibility phenotype of the rest of the progeny couldn't be determined because some trees showed male and/or female sterility and others died before evaluation.

The self-incompatibility and self-compatibility phenotypes of the 59 trees were compared with their S-RNase RFLP patterns. Assuming that clones that have no less than three functional-type *S*-haplotypes are self-incompatible, among the five *S*-haplotypes (S^4, S^6, S^a, S^b, and S^c), three *S*-haplotypes (S^4, S^6, and S^b) seemed to be functional. The other two *S*-haplotypes (S^a and S^c) seemed to be non-functional.

Among six RFLP types observed, only one type, $S^4S^6S^b$, is the group to which only self-incompatible individuals belong and the other three RFLP types, $S^4S^aS^c$, $S^4S^aS^bS^c$, and $S^4S^6S^aS^c$, are the groups to which only self-compatible individuals belong (Table 3.6). All these observations are consistent with the assumption that S^4, S^6, and S^b are functional haplotypes and S^a and S^c are non-functional. Furthermore, both self-incompatible and self-compatible individuals are present in the RFLP type, $S^4S^6S^a$ (Table 3.6). Two *S*-haplotype combinations could represent $S^4S^6S^a$ RFLP type; $S^4S^4S^6S^a$ and $S^4S^6S^aS^a$ depending on which *S*-haplotype, S^4 or S^a, is in double dose. Based on the assumption that clones that have at least three functional *S*-haplotypes are self-incompatible, the former combination would be self-incompatible and the latter would be self-compatible. The genetic control of self-incompatibility and self-compatibility in tetraploid sour cherry seems to involve several functional and non-functional *S*-haplotypes. Therefore, an understanding of the pollen *S*-gene(s) in sour cherry is likely to be crucial to the understanding of self-incompatibility trait in sour cherry. Since certain progeny was not consistent with the hypothesis described above, modifier genes may also have a role in modulating the interaction between the pollen-*S* products and the S-RNases. Modifier genes have been demonstrated to be required for normal self-incompatibility function in *Nicotiana* (McClure et al. 1999) and *Lycopersicon* (Kondo et al., 2002).

Table 3.6. RFLP types of *S*-haplotypes and self-(in)compatibility phenotypes (SI/SC) in the F_1 progeny derived of 'RS'×'EB' mapping population

RFLP type (*S*-haplotype)[Z]	No. of trees investigated	No. of trees SI	SC	?
RFLP type that includes only self-compatible offspring				
$S^4S^aS^c$ ($S^4S^4S^aS^c$ or $S^4S^aS^aS^c$)	21	0	17	4
$S^4S^aS^bS^c$ ($S^4S^aS^bS^c$)	8	0	5	3
$S^4S^6S^aS^c$ ($S^4S^6S^aS^c$)	7	0	6	1
RFLP type that includes only self-incompatible offsprings				
$S^4S^6S^b$ ($S^4S^4S^6S^b$)	8	7	0	1
RFLP type that includes both self-compatible and -incompatible offsprings				
$S^4S^6S^a$ ($S^4S^4S^6S^a$ or $S^4S^6S^aS^a$)	13	6	5	2
$S^4S^aS^b$ ($S^4S^4S^aS^b$ or $S^4S^aS^aS^b$)	2	1	1	0

[Z] S^4, S^6: *S*-haplotypes from sweet cherry (*Prunus avium*)
S^a, S^b, S^c: novel *S*-haplotypes in sour cherry (*Prunus cerasus*)
The *S*-haplotypes that are functional both in pollen and pistil on pollen-pistil recognition of incompatibility reaction are designated in bold face.

3.4. Summary

In this chapter, S-RNases are shown to be present both in styles of self-incompatible and self-compatible cultivars and selections of tetraploid sour cherry (*Prunus cerasus* L.). The cDNAs encoding the S-RNases were cloned from 'Erdi Botermo' and 'Rheinische Schattenmorelle'. The deduced amino acid sequences revealed that 'Erdi Botermo' has the S^4-RNase of sweet cherry (*Prunus avium* L.) in addition to a novel S-RNase (named S^a-RNase in this study), while 'Rheinische Schattenmorelle' has the S^6- and S^a-RNases and two other novel S-RNases (named S^b-, S^c-RNases in this study). These novel S-RNases contained two active sites of T2/*S* type RNases and five regions conserved among other *Prunus* S-RNases. Furthermore, the S^b-RNase mapped to the expected position on *Prunus* linkage group 6 indicating that this linkage relationship is maintained among sour cherry, sweet cherry and almond.

The cessation of 'Crisana' ($S^1S^4S^d$) pollen tube growth in the styles of 'Rainier' (S^1S^4) suggested that a match between any stylar S-RNase and either of the two *S*-alleles in the 2x pollen causes an incompatible interaction. This assumption was supported by the fact that none of the offspring contained the 'EB'-derived S^{6m} haplotype in the 'Rheinische Schattenmorelle' ($S^aS^bS^cS^6$) × 'Erdi Botermo' ($S^aS^4S^4S^{6m}$) cross. These observations seemed to be contradict to the hypothesis that pollen containing two different pollen *S*-alleles loses its self-incompatibility phenotype due to a competition between the pollen *S*-alleles, thus any pollen containing two different *S*-alleles is compatible with any plant indicated by many reports (Chawla et al., 1997; Entani et al., 1999; Golz et al., 1999, 2001; Lewis, 1947).

The self-incompatibility phenotypes were compared with the *S*-haplotypes of the 59 progeny of the cross 'Rheinische Schattenmorelle' × 'Erdi Botermo'. Assuming that clones that have no less than three functional *S*-haplotypes are self-incompatible, three *S*-haplotypes (S^4, S^6, and S^b) seemed to be functional among the six *S*-haplotypes (S^4, S^6, S^{6m}, S^a, S^b, and S^c). The genetic mechanism of self-incompatibility trait in tetraploid sour cherry seems to involve the existence of mutations in the S-RNase or pollen *S*-gene of a number of *S*-haplotypes.

Chapter 4
Identification of stylar non-S-RNases of sweet cherry: A possible ancestral form of S-RNases in *Prunus*

4.1. Introduction

In Chapters 1 and 3, it has been shown that an S-RNase based self-incompatibility system is present in sweet and sour cherries which belong to the subfamily Amygdaloideae of the Rosaceae. Although Amygdaloideae and Maloideae to which apple and pear belong are the subfamilies of Rosaceae, the S-RNases of Amygdaloideae and Maloideae showed only about 30 % identity with each other. In addition, the genomic structures of S-RNases were different in that amygdaloideous S-RNase genes have two introns whereas maloideous S-RNase genes have one of the two. These findings may imply relatively distant evolutionary relationship between S-RNases of Amygdaloideae and Maloideae.

Non-S-RNases (syn. S-like RNases) that are T2/*S* type RNases (Bariola and Green, 1997) but not associated with self-incompatibility have been identified from various plant species. In Chapter 1, basic protein spots (non-S-protein spots) that were reactive with the antiserum raised against S^4-RNase of Japanese pear in the 2D-PAGE profile of stylar proteins of sweet cherry (*Prunus avium*) were found. These non-S-proteins are supposed to be RNase.

In this chapter, non-S-proteins were further characterized to be non-S-RNase in the pistil of sweet cherry. Based on the genomic structure and phylogenetic placement of S-RNases and the non-S-RNase, evolutional background of self-incompatibility and S-RNase molecules in *Prunus* species were discussed.

4.2. Materials and Methods

Plant material

Young leaves and flower buds at the balloon stage of development of the sweet cherry (*Prunus avium* L.) cultivar 'Satonishiki' were collected. We also collected young leaves from seven other sweet cherry cultivars: 'Bing', 'Burlat', 'Early Rivers',

'Hinode (Early Purple)', 'Moreau', 'Nanyo', and 'Van'. Two other sweet cherry cultivars, 'Hedelfingen' and 'Mona' and one sour cherry (*Prunus cerasus*) cultivar 'Crisana' were kindly provided by Prof. A. Iezzoni, Michigan State Univ., U.S.A. We also collected young leaves from Japanese plum (*Prunus salicina* 'Santa Rosa' and 'Sordum'), Japanese apricot (*Prunus mume* 'Nanko'), peach (*Prunus persica* 'Shimizuhakuto'), apple (*Malus* × *domestica* 'Fuji'), and Japanese pear (*Pyrus pyrifolia* 'Housui').

Amino acid sequencing

Styles with stigmas were dissected from flower buds of 'Satonishiki', frozen immediately in liquid nitrogen, and stored at –80 °C until used. Acetone powder was prepared for the styles with stigmas as described in Chapter 1. Crude extracts from the acetone powder were subjected to 2D-PAGE using NEPHGE in the first dimension and SDS-PAGE in the second dimension as described in Chapter 1 with slight modifications. After NEPHGE, the first dimensional gels were incubated with the solution consisting of 10 % TCA and 5 % sulfosalicylic acid for 1 hr to remove carrier ampholite [Ampholine, Pharmalyte (Amersham Bioscience, Tokyo, Japan)] from the gels because carrier ampholite reacts with Coomassie Brilliant Blue to make an insoluble precipitation in the gels. After incubation, the gels were washed gently by double distilled water, equilibrated with the SDS sample buffer for 15 min, and subjected to SDS-PAGE. Then, proteins in the gel were detected by CBB-R250. To determine the internal amino acid sequences, the CBB-R250 - stained non-S-RNase spots were digested with trypsin in the gel (Hellman et al. 1995). The digested peptides were recovered from the gel and separated by the SMART-System (Amersham Biosciences, Tokyo, Japan). Several purified peptide fractions obtained were dotted on ProSorb (Applied Biosystems, Tokyo, Japan) and subjected to a gas-phase protein sequencer (476A, Applied Biosystems, Tokyo, Japan).

cDNA library construction and screening

cDNA library was constructed from total RNA isolated from styles with stigmas of sweet cherry cultivar 'Satonishiki' at the balloon stage of development as described in Chapter 1.

Degenerate primer (5'- ACNAGYTGGCCNAAYCTNGA -3'; where N=A or C or G or T; Y=C or T) designed based on the fragment A34 (TSWPNLE) from the non-S-RNase spots was used in 3' RACE under the PCR condition identical to that described in Chapter 1. The PCR products were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, Wis.). DNA sequences of the inserts of several clones were determined using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

The insert of putative 3'RACE clones for non-S-RNases obtained as described above were labeled by random primer incorporation of DIG-dUTP (Roche, Tokyo, Japan) and used as probes to screen the primary cDNA library consisting of 3×10^6 plaque forming units. The cDNA library was screened as described in Chapter 1, selected clones were converted to pBluescript plasmids, and DNA was sequenced as described above.

Construction of a phylogenetic tree

Amino acid sequences of 42 T2/*S* type RNases were used for construction of a phylogenetic tree: one non-S-RNase from sweet cherry identified in this study, eight S-RNases from the rosaceous subfamily Amygdaloideae, six S-RNases from the rosaceous subfamily Maloideae, eight S-RNases from Asteridae, five basic and 13 acidic non-S-RNases of higher plants. The alignment begins at the first mature peptide, and sequences were terminated at the last conserved cysteine residue of S-RNases because excessive sequence divergence downstream of this site rendered the remaining sequence unalignable as suggested by Igic and Kohn (2001). The amino acid sequences were aligned using computer software CLUSTAL X (Thompson et al., 1997) and the neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic reconstruction.

RNA blot analysis

Styles with stigmas, ovaries, petals, calyxes, peduncles, and anthers were dissected from the flower buds at the balloon stage of development. Young leaves were collected in spring. Cotyledons of dissected embryos from 'Satonishiki' seeds, and

stems and roots from 'Satonishiki' seedlings obtained after 6 weeks of germination in vermiculite were also used. To examine whether the expression of non-S-RNase is related to the phosphate starvation, in vitro shoots of 'Satonishiki' were used. They were cultured and maintained on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L BA, 0.1 mg/L IBA, and 30 g/L sucrose and solidified with 0.8 % agar. The phosphate starvation treatment was initiated by subculturing the shoots to the same fresh medium but without phosphate (MS-Pi medium) as described by Ma and Oliveira (2000). Six to 13 days after subculturing on MS-Pi medium, the leaves from the shoots cultured were collected. All the tissues collected were frozen immediately in liquid nitrogen, and stored at –80 °C until used. Total RNA was isolated from these samples with the cold phenol method and RNA blot analysis was performed as described in Chapter 1.

Cloning and DNA sequencing of PCR-amplified fragments

Two primers, SL-T2 (5'- TCCTTCTTGTGCTCTTTTCAGC -3') as a forward primer and SL-C5R (5'- GTGTCGTCATAGCAAAGTACAAC -3') as a reverse primer, were designed based on the deduced amino acid sequences from the cDNA described above. SL-T2 primer sequences correspond to LLLVLFSA and SL-C5R primer correspond to VVLCYDDT, which correspond to the signal peptide and C5 conserved domain of PA1 cDNA, respectively. The PCR condition was identical to that described in Chapter 1. PCR products were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, WI) and their sequences were determined as described above.

DNA blot analysis

Total DNA was isolated from young leaves by the CTAB method as described by Stockinger et al. (1996). Genomic DNA blot analysis was performed as described in Chapter 1. The cDNAs from non-S-RNase and S^4-RNase were PCR labeled with DIG-dUTP (Roche, Tokyo, Japan) and used as probes as described in Chapter 1. Two different stringency wash conditions, high (2 × 5 min at room temperature with 2 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 °C with 0.1 × SSC and 0.1 % SDS) and low (2 × 5 min at room temperature with 2 × SSC and 0.1 % SDS followed by 2 ×

15 min at 60 °C with 0.1 × SSC and 0.1 % SDS), were used.

4.3. Results

Amino acid sequencing

Internal amino acid sequencing was conducted with two non-S RNase spots, PA1 (*Prunus avium* 1) and PA2 (Fig. 4.1), that were reactive with the antiserum raised against Japanese pear S-RNase (Tao et al., 1999b). After trypsin digestion, six fragments each were obtained for PA1 and PA2 by the SMART-System (Table 4.1, Fig. 4.2).

Fig. 4.1. S-RNases and non-S-RNases of a sweet cherry cultivar, Satonishiki. Stylar proteins from 'Satonishiki' were separated by 2D-PAGE and visualized by silver staining. Two non-S-RNases (PA1 and PA2) and S-RNases are indicated by arrows.

Table 4.1. Amino acid sequences of internal fragments of PA1 and PA2.

	fragment No.	amino acid sequences
PA1	A 25-1	FSSQNTYLSK
	A 25-2	QTNCGTDIY
	A 30	ACDLWEENK
	A 32	SSPLLLxHR
	A 34	TSWPNLEQR
	A 51	DNDMWFWAMEYEK
PA2	B 17	MIPGIKNIY
	B 19	FSSQNTYLSK
	B 23	ACDLFE
	B 25	TSWPNLEQR
	B 32	KPEF
	B 40	DND

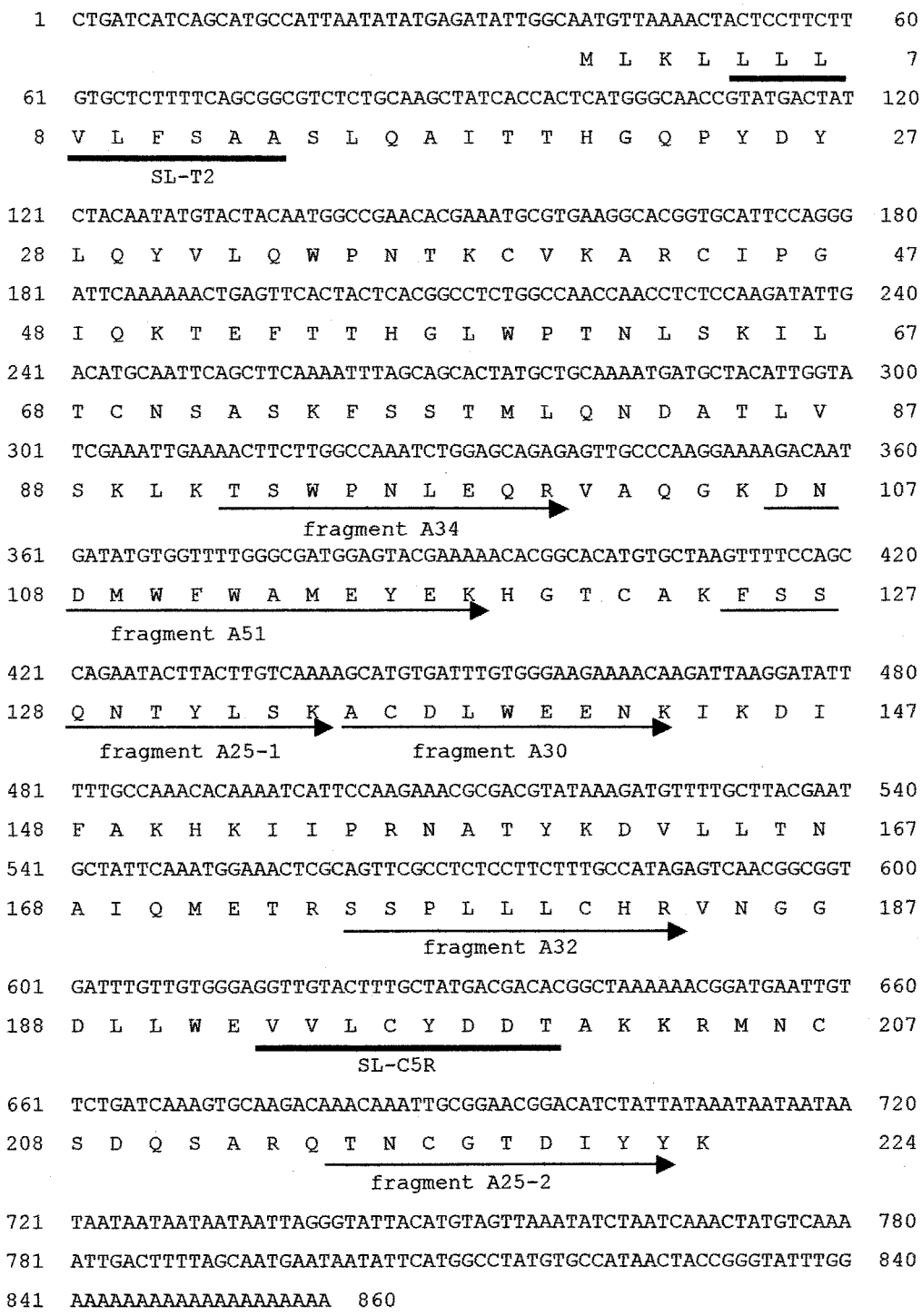

Fig. 4.2. The nucleotide and deduced amino acid sequences of PA1 cDNA. The internal amino acid sequences obtained with the trypsin digestion are indicated by arrows. The location of two primers, SL-T2 and SL-C5R, in the PA1 cDNA sequence were underlined.

cDNA cloning and sequencing of RNase PA1

Several 3'RACE clones were obtained and sequenced. The deduced amino acid sequences from the clones contained the sequences of several PA1 internal fragments including A25-1 (FSSQNTYLSK), A25-2 (QTNCGTDIY), A30 (ACDLWEEN), A32 (SSPLLLCHR), and A51 (DNDMWFWAMEYEK) (Table 4.1, Fig. 4.2). Several cDNA clones were obtained when the 3'RACE clone was used as a probe to screen the pistil cDNA library. The deduced amino acid sequences contained the amino acid sequences of six internal fragments from PA1, suggesting that all of them encoded PA1 (Fig. 4.2). cDNA for PA2 was not obtained. The deduced amino acid sequences contained two active domains of the T2/*S* type RNases and showed that the cDNA clones obtained encode T2/*S* type RNases (Fig. 4.3). PA1 appeared to be extracellular protein with the putative N-terminal secretion signal of hydrophobic amino acid sequences (Fig. 4.3). Based on the (-3, -1) rule (von Heijne, 1986), the peptide cleavage site was presumed to be located between Gln-16 and Ala-17 (Fig. 4.3). The predicted mature protein of PA1 consists of 208 amino acids with a molecular weight of 23.9 kDa and pI value of 8.65, which is consistent with the results obtained by 2D-PAGE analysis of stylar proteins (Figs. 4.1, 4.3).

The deduced amino acid sequence of RNase PA1 was compared with other sequences in several databases using the BLAST search procedure. It appeared that PA1 had the highest sequence identity of 30 % with S^3-RNase of sweet cherry, which was associated with self-incompatibility in sweet cherry (Table 4.2). Moreover, RNase PA1 has 20 to 30 % sequence identity with rosaceous and solanaceous RNases and other non-S-RNases including the acidic and the basic proteins (Table 4.2).

Table 4.2. Amino acid sequence identities (%) of S- and non-S-RNases from sweet cherry with plant T2/*S* type RNases.

	class III RNases[z]							class I, II RNases[z]					
	rosaceous S-RNases				basic non-S			acidic non-S of Rosaceae			acidic non-S of Asteridae		
	PA-S3	PD-Sc	MD-Sf	PP-S4	LC1	MC	X2	PD1	PD2	PP-nonS	NE	RNS1	RNS2
PA1	30	26	22	21	23	27	27	21	20	24	26	26	24

[z] T2/*S* type RNases were classified into three RNases, class I, II, and III by Igic and Kohn (2001).

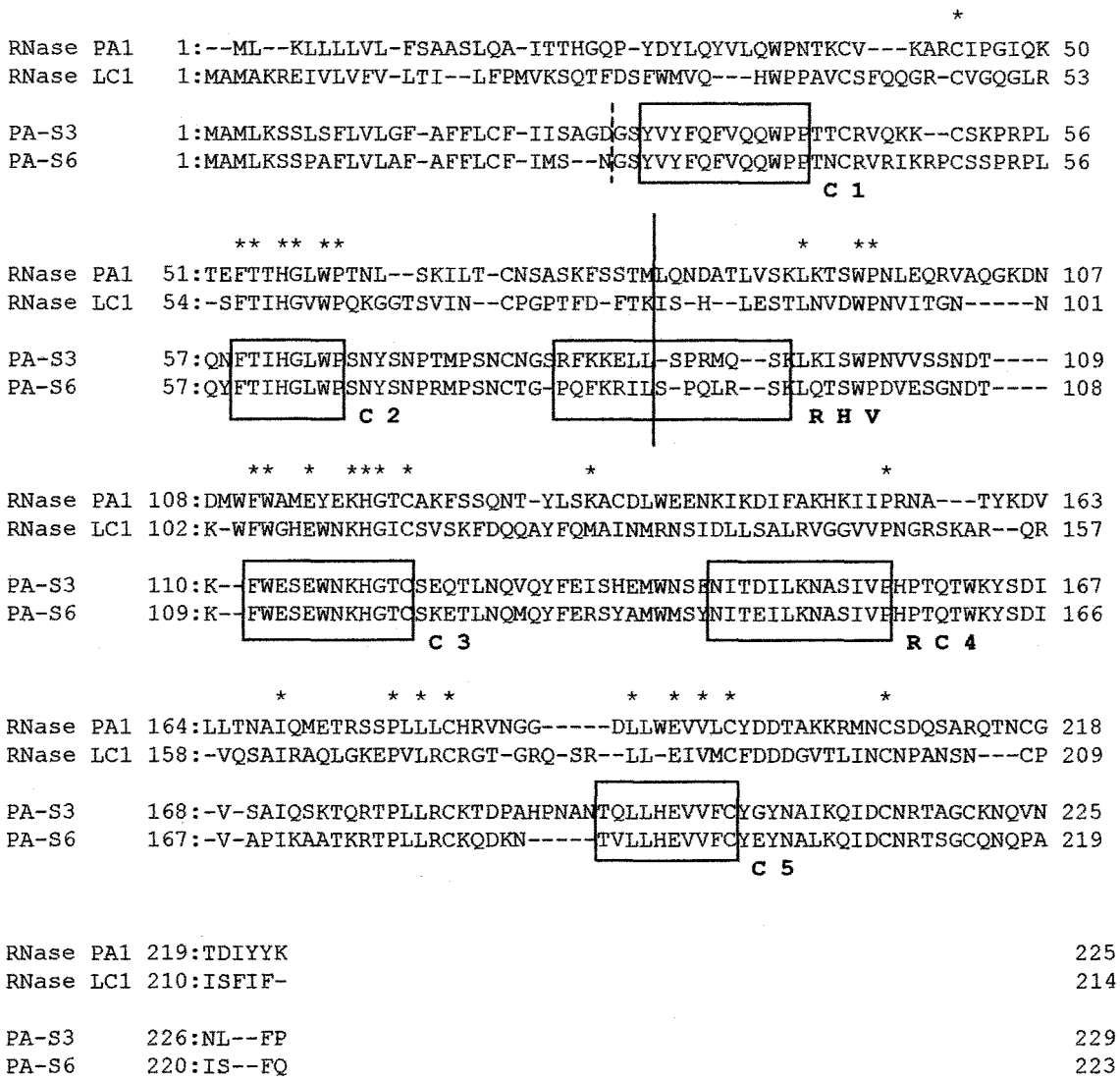

Fig. 4.3. Amino acid sequence alignment of two basic non-S-RNases, PA1 and LC1, and two sweet cherry S-RNases, S^3 and S^6. The alignment was generated by CLUSTAL X (Thompson et al., 1997). Gaps are marked by dashes. The five conserved regions, C1, C2, C3, RC4, C5 of rosaceous S-RNases (Ushijima et al., 1998) are boxed. Conserved residues in all RNases are indicated by *asterisks*. The intron insertion sites of PA1 and LC1, and the second intron insertion site of *Prunus* S-RNases are indicated by vertical line. The first intron insertion sites of *Prunus* S-RNases are indicated by vertical dotted line. Sequence data for the RNases included are as follows: RNase PA1 from sweet cherry [*Prunus avium* 'Satonishiki' (this study)], PA-S3, S6 [S^3- and S^6-RNases (DDBJ/EMBL/Gen-Bank Accession Nos. AB010305 and AB010306, respectively) (Tao et al., 1999b)], RNase LC1 [basic non S-RNase of *Luffa cylindrica* (D64011)].

As shown in Fig. 4.4, a phylogenetic tree with 41 T2/*S* type RNases was generated by the neighbor-joining method (Saitou and Nei 1987) after the alignment. Igic and Kohn (2001) classified the T2/*S* type RNases into three groups based on the sequence similarity and intron structure. The acidic RNases were divided into two groups, class I with less than four introns and class II with more than four introns, whereas the basic non-S-RNases and S-RNases were classified to class III RNase (Igic and Kohn, 2001). Eight amygdaloideous S-RNases analyzed in this study formed one subgroup in the group of rosaceous S-RNases, which are classified into class III RNases as is the case with Igic and Kohn (2001) (Fig. 4.4).

As described in Chapter 1, similarities of amino acid sequences between amygdaloideous and maloideous S-RNases were very low, about 30 % although they belong to the same family, Rosaceae. Consistent with this finding, the S-RNases of Rosaceae were further divided into two subgroups, S-RNases of Amygdaloideae and Maloideae in the phylogenetic tree. The species - specific subgroups were not found in the subgroup of Amygdaloideae, which supports the hypothesis that the S-RNases of Rosaceae have diverged after the divergence of subfamilies but before the divergence of species (Igic and Kohn, 2001; Ushijima et al., 1998). Although RNase PA1 was classified to class III RNase and more similar to the S-RNases of *Prunus* than to a number of related plant RNases, it is located distinct from the clade of S-RNases of *Prunus* in the phylogenetic tree.

Expression of RNase PA1

A hybridization signal with RNase PA1 cDNA at about 900 bp was detected only with the total RNA isolated from styles with stigmas (Fig. 4.5). The total RNAs from cotyledons of embryos in seeds, stems and roots of seedling, calyx, peduncles, petals, anthers, ovaries, and leaves gave no hybridization signal (Fig. 4.5). These results indicated the genes for PA1 was specifically expressed in the style. The hybridization signal was not detected with total RNAs from the leaves of six and 13 days after subculturing on the medium without phosphate, indicating the expression of PA1 was not associated with phosphate starvation (data not shown).

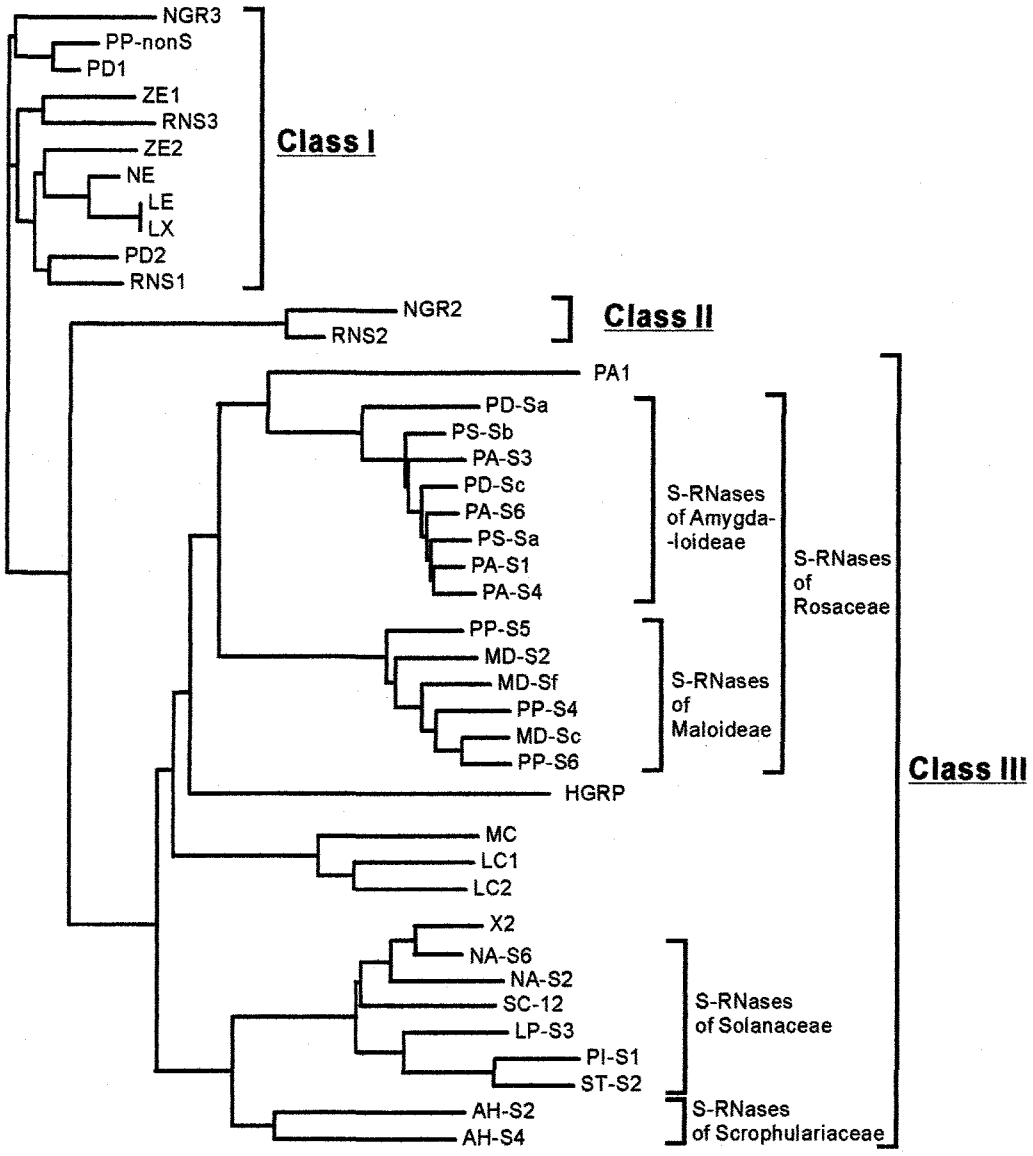

Fig. 4.4. Phylogenetic tree of the T2/*S* type RNases. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on an alignment of 41 plant T2/*S* type RNases. All T2/*S* type RNases used were classified into three classes, Class I, II, and III, based on Igic and Kohn (2001). Sequence data for 20 non-S-RNases included as follows: PA1 of *Prunus avium*; NE of *Nicotiana tabacum* (DDBJ/EMBL/Gen-Bank Accession Nos. U13256); NGR2 and NGR3 of *Nicotiana glutinosa* (AB032256 and AB032257); RNS1, RNS2, and RNS3 of *Arabidopsis thaliana* (U05206, M98336 and U05207); LE and LX of *Lycopersicon esculentum* (S33840 and S53507); ZE1 and ZE2 of *Zinnia elegans* (U19923 and U19924); PD1 and PD2 of *Prunus dulcis* (AF227522 and AF157011); PP-nonS of *Pyrus pyrifolia* (JC4867); HGRP of *Pisum sativum* (Y11824); LC1 and LC2 of *Luffa cylindrica* (D64011 and D64012); MC of *Momordica charantia* (P23540); X2 of *Petunia inflata* (S28611). Sequence data for eight S-RNases of Amygdaloideae included as follows: PA-S1, S3, S4, and S6 [S^1, S^3, S^4, and S^6-RNases of sweet cherry (*Prunus avium*) (AB028153, AB010306, AB028154, and AB010305, respectively)]; PS-Sa and Sb [S^a and S^b-RNases of Japanese plum (*P. salicina*) (AB026981 and AB026982)]; PD-Sa and Sc [S^a and S^c-RNases of almond (*P. dulcis*) (AB026836 and AB011470)]. Sequence data for six S-RNases of Maloideae included as follows: PP-S4, S5 and S6 [S^4, S^5, and S^6-RNases of Japanese pear (*Pyrus pyrifolia*) (AB009385, D88282, and AB002142)]; MD-S2, Sc and Sf [S^2, S^c, and S^f-RNases of apple (*Malus x domestica*) (U12199, D50836,and D50837)]. Sequence data for six S-RNases from Solanaceae included as follows: NA-S2, S6 [S^2 and S^6-RNases of *Nicotiana alata* (U08860 and U08861)]; LP-S3 [S^3-RNase of *Lycopersicon peruvianum* (X76065)]; PI-S1 [S^1-RNase of Petunia inflata (S20989)]; SC-12 [(S^{12}-RNase of Solanum tuberosum (X62727)]. Sequence data for two S-RNases from Scrophulariaceae included as follows: AH-S2, S4 [S^2 and S^4-RNases of *Antirrhinum hispanicum* (X96465 and X96466)].

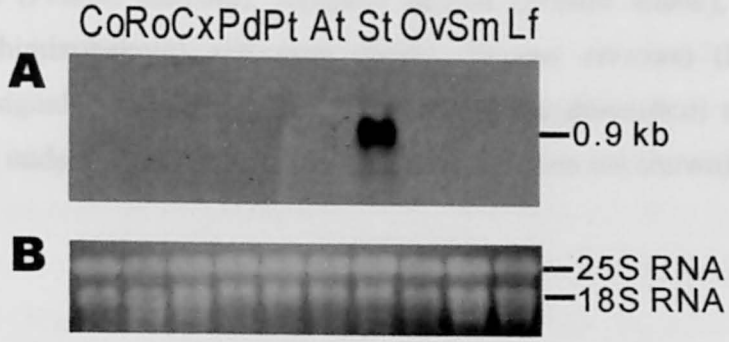

Fig. 4.5. RNA blot analysis of total RNA from various tissues of sweet cherry 'Satonishiki' and 'Satonishiki' seedlings. Cotyledon (Co), root (Ro), and stem (Sm) are from 'Satonishiki' seedling, and calyx (Cx), peduncle (Pd), petal (Pt), anther (At), ovary (Ov), and styles with stigmas (St) are from flower buds of 'Satonishiki' at the balloon stage of development and leaf (Lf) is from 'Satonishiki'. Photograhs show the blot hybridized to the PA1 cDNA (A) and ethidium bromide staining of the gel (B).

Structure of the RNase PA1 gene

Comparisons of the DNA sequences of the PA1 cDNA and the genomic DNA fragment amplified with the SL-T2 and SL-C5R primers revealed that a sole intron is present in the DNA sequence encoding Met-79 and Leu-80 of the PA1 gene (Fig. 4.3). The position is similar to the position where the intron of maloideous and solanaceous S-RNase genes and second intron of amygdaloideous S-RNase genes is located (Fig. 4.3). Intron was not found in the PA1 gene where the first introns of amygdaloideous S-RNase genes are located.

Four to six hybridization signals with the PA1 probe depending on the restriction endonucleases used were observed with the genomic DNA blot analysis for 'Satonishiki' sweet cherry under a low stringency wash condition (Fig. 4.6A). Under a high stringency wash condition, 2.0 kb, 6.4kb, and 12.1 kb bands were observed with *Dra*I, *Eco*RI, and *Hin*dIII digestions, respectively (Fig. 4.6B).

Ten sweet cherry cultivars with different *S*-haplotypes were subjected to DNA blot analysis after *Dra*I digestion (Fig. 4.6C). Under a low stringency wash condition, three or four bands were observed for each cultivar. Less polymorphism was observed when probed with the PA1 probe than with the S-RNase probe (Fig. 4.6C, D).

Hybridization signals with the PA1 probe were found in the genomic DNA blot of Japanese plum (*Prunus salicina*), Japanese apricot (*Prunus mume*), peach (*Prunus persica* cv. Shimizuhakuto) and sour cherry (*Prunus cerasus*) (Fig. 4.6E). No hybridization signal was observed with apple (*Malus x domestica*) and pear (*Pyrus pyrifolia*) even under a low stringency wash condition (data not shown).

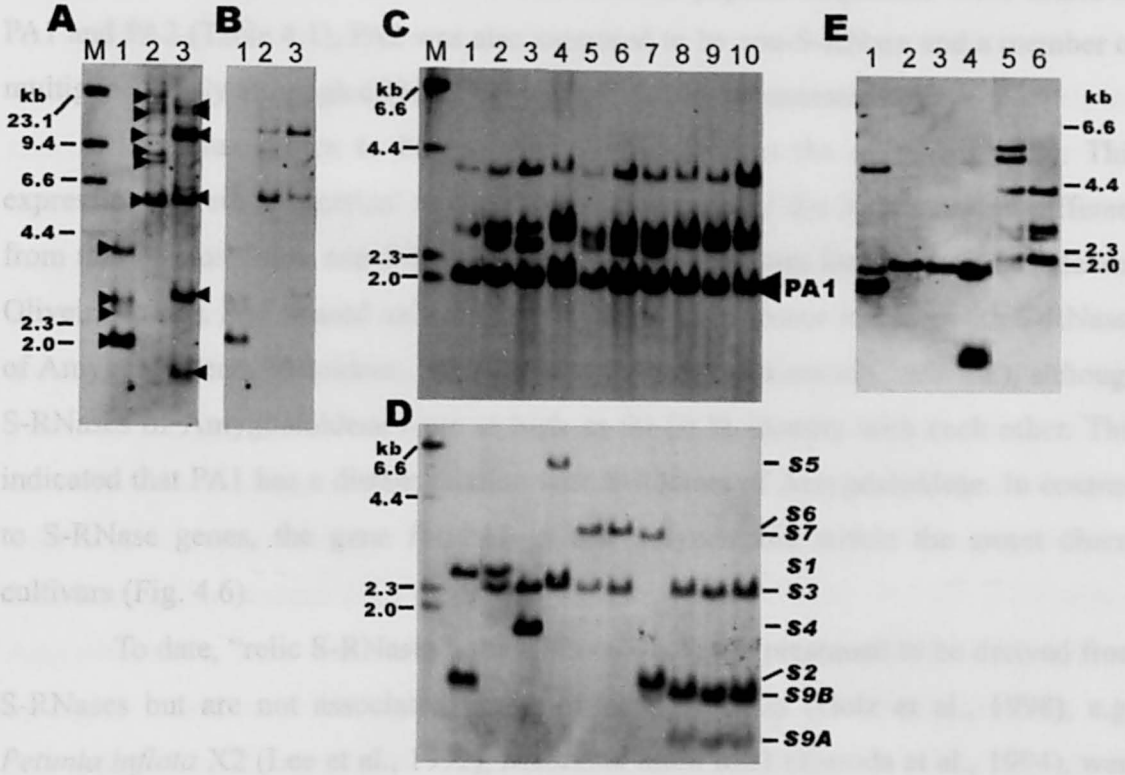

Fig. 4.6. Genomic DNA blot analysis using the cDNA encoding PA1 of sweet cherry as probes. The blot of the digested DNA of 'Satonishiki' with *Dra*I, *Eco*RI, and *Hin*dIII was hybridized with PA1 cDNA under low stringency wash (see text) (A) or high stringency wash (B). The blot from ten sweet cherry cultivars digested with *Dra*I was hybridized with PA1 cDNA (C) or S^4-RNase cDNA (D). The blot from the DNAs of five species of Rosaceae digested with *Dra*I was hybridized with PA1 cDNA (E). A and B: (M) Lambda *Hin*dIII DNA marker, (1) *Dra*I digest, (2) *Eco*RI digest, (3) *Hin*dIII digest. C and D: (M) Lambda *Hin*dIII DNA marker, (1) 'Early Rivers' (S^1S^2), (2) Van (S^1S^3), (3) 'Bing' (S^3S^4), (4) 'Hedelfingen' (S^3S^5), (5) 'Nanyo' (S^3S^6), (6) 'Satonishiki' (S^3S^6), (7) 'Hinode' (S^2S^7), (8) 'Burlat' (S^3S^9), (9) 'Mona' (S^3S^9), (10) 'Moreau' (S^3S^9). E: (1) *Prunus persica* 'Shimizuhakuto', (2) *P. salicina* 'Sordum', (3) *P. salicina* 'Santa Rosa', (4) *P. mume* 'Nanko', (5) *P. cerasus* 'Crisana', (6) *P. avium* 'Satonishiki'.

4.4. Discussion

Although two non-S-RNases (PA1 and PA2) were identified by 2D-PAGE analysis (Fig. 4.2), only a cDNA encoding PA1 was obtained from the stylar cDNA library (Fig. 4.1, 4.2 and 4.3). The amino acid sequencing analysis confirmed that the gene for PA1 encodes extracellular basic T2/*S* type RNases (Fig. 4.1 and 4.3). The genomic DNA blot analysis indicated that PA1 is a member of multigene family of non-S-RNases (Fig. 4.6). Because similar internal peptide sequences were found in PA1 and PA2 (Table 4.1), PA2 was also supposed to be non-S-RNase and a member of multigene family although cDNA cloning for PA2 was unsuccessful.

PA1 was shown to be specifically expressed in the style (Fig. 4.5). This expression pattern is identical to the expression pattern of the S-RNases but different from that of most other non-S-RNases whose expression was found in leaves (Ma and Oliveira, 2000). PA1 shared only about 20 % to 30 % sequence identity with S-RNases of Amygdaloideae, Maloideae, Solanaceae, and Scrophulariaceae (Table 4.2), although S-RNases of Amygdaloideae have as high as 60-80 % identity with each other. This indicated that PA1 has a distant relation with S-RNases of Amygdaloideae. In contrast to S-RNase genes, the gene for PA1 is less polymorphic within the sweet cherry cultivars (Fig. 4.6).

To date, "relic S-RNases", the RNases which are presumed to be derived from S-RNases but are not associated with self-incompatibility (Golz et al., 1998), e.g., *Petunia inflata* X2 (Lee et al., 1992), *Nicotiana alata* MS1 (Kuroda et al., 1994), were found in the Solanaceae that has an S-RNase-mediated self-incompatibility system as does Rosaceae. We compared biochemical and physiological characters, and gene structure of PA1 with relic S-RNases, RNase X2. Although PA1 resembled RNase X2 in that it is a basic RNases and specifically expressed in the style, the phylogenetic placement of PA1 and RNase X2 was quite different. PA1 was placed distinct from S-RNases of Rosaceae while RNase X2 is well incorporated in the clade of S-RNases of the Solanaceae in the phylogenetic reconstructions (Golz et al, 1998; Richman et al., 1997). Furthermore, in contrast to RNase X2, PA1 constitutes a multigene family (Fig. 4.6). Taking this into consideration, we propose that PA1 and RNase X2 have different evolutional history as described below.

The presence of only a single intron in the PA1 gene (Fig. 4.3) may have significant implications for the evolutional relationship between PA1 and S-RNases of Rosaceae. Recently, Igic and Kohn (2001) reported the phylogenetic reconstruction of plant T2/*S* type RNases with taking the intron numbers into consideration. The S-RNases of Rosaceae, Scrophulariaceae and Solanaceae, and all the other basic non-S-RNases, such as LC1 and LC2 from *Luffa cylindrica* and HRGP from *Pisum sativum*, were classified into class III RNases. Except for S-RNases in Amygdaloideae, all the class III RNase genes have only a single intron at the same position. Amygdaloideous S-RNase genes have additional intron that is located at the junction of the sequence for a signal peptide and mature protein (Ma and Oliveira, 2001; see chapter 2). If the S-RNases of Rosaceae, Scrophulariaceae, and Solanaceae have the same origin, we would expect that the ancestral form of S-RNase genes in Amygdaloideae to have only a single intron as with other S-RNase genes. Considering the intron number and phylogenetic placement, PA1 could be a possible candidate of ancestral form of S-RNases in Amygdaloideae. We hypothesized that various S-RNase genes of Amygdaloideae were diverged from an ancestral S-RNase gene that is a member of the PA1 multigene family. Namely, PA1 is different from RNase X2 in that S-RNases of Amygdaloideae originated from PA1, while RNase X2 originated from S-RNases of Solanaceae (Igic and Kohn, 2001). The hypothesis that the S-RNases of Amygdaloideae were evolved from PA1 and several kinds of *S*-haplotypes were diverged, may imply that ancestral species of Amygdaloideae and Rosaceae were self-compatible. This might be addressed when the function of PA1 is fully elucidated.

The fact that PA1 orthologs appeared to be present universally in amygdaloideous species (Fig. 4.6E) might indicate that PA1 is not simply a relic form of S-RNase but has an important physiological function. Murfett et al. (1996) conducted plant transformation experiments with S-RNase genes and suggested that S-RNases act as factors controlling interspecific pollen rejection. Since PA1 shares similarities in genomic structure and expression pattern with S-RNases, it could be involved in interspecific pollen rejection. Some non-S-RNases, such as RNS 2 from *Arabidopsis thaliana* and RNase LE from *Lycopersicon esculentum*, are known to play a role in response to phosphate limitation by scavenging phosphate from RNA (Loffler et al., 1993; Taylor et al., 1993). Some non-S-RNases, such as ZRNase II from *Zinnia*

elegans and RNase NW from *Nicotiana glutinosa*, are known to be involved in wound response and disease resistance (Ye and Droste, 1996; Kariu et al., 1998). Other non-S-RNases, such as RNS1 and RNase NE from *Nicotiana tabacum*, are known to respond to both phosphate limitation and disease infection (Bariola et al., 1994; Galiana et al., 1997; Hugot et al., 2002; LeBrasseur et al., 2002). Since PA1 expression is specific to the style (Fig. 4.5) and does not respond to phosphate starvation treatment, the possibility that it could be associated with a plant defense system against disease is intriguing. If PA1 is involved in a disease resistance function, the gene is the most likely candidate for the ancestor of the amygdaloideous S-RNases since self-incompatibility is speculated to be evolved from the plant defense system that involves a cell-to-cell recognition.

To date, several lines of research to identify pollen-*S* gene by RNA differential display (Li et al., 2000; McCubbin et al., 2000a), chromosome walking (McCubbin et al., 2000b; Ushijima et al., 2001), and two-hybrid system (Sims and Ordanic, 2001) were reported. However, pollen *S*-gene(s) remains to be determined. The evolutional origin of S-RNase and gametophytic self-incompatibility system within the Rosaceae might be addressed when the pollen-*S* gene is fully elucidated in the Rosaceae.

4.5. Summary

Although Amygdaloideae and Maloideae belong to the same family Rosaceae, the S-RNases of Amygdaloideae and Maloideae showed only about 30 % identity with each other as described in Chapter 1. In addition, the genomic structures of S-RNases were different in that amygdaloideous S-RNase genes have two introns whereas maloideous S-RNase genes have one of the two. These findings may imply a relatively distant evolutionary relationship between S-RNases of Amygdaloideae and Maloideae. To further analyze the evolutionary relationship of amygdaloideous and maloideous S-RNases, T2/*S* type RNase, other than S-RNases in Amygdaloideae was characterized, in this chapter.

The amino acid sequencing analysis confirmed that the gene for non-*S*-protein (PA1) found in Chapter 1 encodes extracellular basic T2/*S* type RNase categorized in class III RNases. PA1 was shown to be specifically expressed in the style, as in the case with S-RNases. In contrast to the S-RNases of Amygdaloideae, however, PA1 gene has only a single intron. Except for S-RNases in Amygdaloideae, all the class III RNase genes have only a single intron at the same position. Amygdaloideous S-RNase genes have an additional intron that is located at the junction of the sequence for a signal peptide and mature protein. If the S-RNases of Rosaceae, Scrophulariaceae, and Solanaceae have the same origin, we would expect that the ancestral form of S-RNase genes in Amygdaloideae that have only a single intron as with other S-RNase genes. Taken the intron number into account, PA1 could be a possible candidate of ancestral form of S-RNases in Amygdaloideae. The phylogenetic placement of PA1 supported this assumption because PA1 is located most closely to but distinct from the clade of S-RNases of *Prunus*. It is possible, therefore, that various S-RNase genes of Amygdaloideae were diverged from an ancestral S-RNase gene that is a member of the PA1 multigene family. The discovery of PA1 reveals that amygdaloideous S-RNases and maloideous S-RNases have different evolutional backgrounds, which may be the reason for the low amino acid sequence similarity between them.

Conclusion and Prospects

This study identifies and characterizes S-RNases that are involved in self-incompatibility of two commercially important cherry species, diploid sweet cherry (*Prunus avium*) and tetraploid sour cherry (*P. cerasus*). The results obtained in this study make it possible to determine *S*-haplotypes and pollen incompatibility groups of cherries without conducting any conventional cross pollination and pollen tube growth tests. Furthermore, a successful cloning of cDNA for S-RNase in this study opens the door for molecular breeding of self-compatibility through antisense RNA and RNAi techniques. This thesis comprises four chapters and the summary of each chapter is as follows.

In Chapter 1, pistil proteins of sweet cherry cultivars were surveyed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to identify pistil *S*-proteins associated with gametophytic self-incompatibility. Glycoprotein spots linked to *S*-haplotypes were found. Based on their N-terminal amino acid sequences, cDNAs for these proteins were cloned. The deduced amino acid sequences revealed that they were S-RNases. Genomic DNA and RNA blot analyses further confirmed that the cDNAs encode S-RNases; thus the pistil *S*-proteins identified by 2D-PAGE are S-RNases.

In Chapter 2, a molecular typing system was developed based on RFLP analysis using S-RNase cDNA as a probe. In addition, a much simpler method of PCR-RFLP analysis was developed to determine *S*-haplotypes of several sweet cherry cultivars. Since vegetative tissues were used in the DNA-based technology for the *S*-typing, *S*-haplotypes of juvenile seedlings could be determined quickly. Although six *S*-haplotypes had been assigned previously, numerous additional *S*-haplotypes have been identified based on the DNA-based analysis. However, the nomenclature contained inconsistencies and redundancies. To resolve this conflict, standard nomenclature with RFLP band sizes for each *S*-haplotypes was provided. This standard nomenclature is currently employed for determining the *S*-haplotypes of sweet cherry cultivars. As shown in Appendix 1, *S*-haplotypes of several cultivars were reconsidered and 22 incompatibility groups were assigned at the 2001 ISHS Cherry Symposium held at Hood River, Oregon and Richland, Washington State, USA.

In Chapter 3, genetic control of self-incompatibility and self-compatibility in tetraploid sour cherry was discussed based on the analysis of *S*-haplotypes and self-incompatibility phenotypes of cultivars and their offspring. Tetraploid sour cherry, which has evolutionally close relation to sweet cherry, includes both self-incompatible and self-compatible cultivars. A crossing experiment revealed that a match between any stylar S-RNase and either of the two *S*-haplotypes in the 2x pollen causes an incompatible interaction in sour cherry. The *S*-haplotypes and self-incompatibility phenotypes of two self-compatible cultivars and their 80 F_1 clones were determined. The relationship between *S*-haplotypes and self-incompatibility phenotypes also suggested that a match between any stylar S-RNase and either of the two *S*-haplotypes in the 2x pollen causes an incompatible interaction. These results are contradictive to the competitive interaction hypothesis that any pollen containing two different *S*-alleles becomes compatible to all *S*-haplotypes. Furthermore, it appeared that mutations in the S-RNase or pollen S-gene of a number of S-haplotypes seemed to be involved in the genetic control of self-incompatibility and self-compatibility in sour cherry.

In Chapter 4, non-S-RNase in the pistil of sweet cherry was identified and characterized. The discovery of non-S-RNase (PA1) has revealed complications about the evolution of S-RNase and self-incompatibility in Rosaceae. The genomic structure and phylogenetic placement of PA1 strongly suggested that PA1 is a possible candidate of an ancestral form of S-RNases in *Prunus*. This indicates that S-RNases of *Prunus* have different evolutional backgrounds from S-RNases of *Malus* and *Pyrus*.

In conclusion, the information obtained in this thesis is very useful not only for researchers but also for growers and breeders of cherries. In addition, the experimental techniques and results described in this thesis should be useful for the study on self-incompatibility of other fruit tree species of *Prunus* such as almond (*Prunus dulcis*), apricot (*P. armeniaca*), European or common plum (*P. domestica*), Japanese plum (*P. salicina*), and Japanese apricot (*P. mume*).

Appendix 1. Sweet cherry incompatibility groups and haplotypes-consensus of research since 1996

Group No.	*S*- haplotypes	Cultivars	References
Group I	*S1S2*	Baumanns May A	Boskovic and Tobutt, 2001
		Bedford Prolific A	Boskovic and Tobutt, 2001
		Black Downton	Boskovic and Tobutt, 2001
		Black Tartarian	Wiersma et al., 2001
		Carnation C	Boskovic and Tobutt, 2001
		Early Rivers	Boskovic and Tobutt, 1996; Tao et al., 1999b; Choi et al., 2000 Wiersma et al., 2001; Hauck et al., 2001
		Emperor Francis B	Boskovic and Tobutt, 2001
		F1/3	Boskovic et al., 1997
		Nanni	Schmidt et al., 1999
		Ronald's Heart	Boskovic and Tobutt, 2001
		Roundel	Boskovic and Tobutt, 1996
		Sparkle	Wiersma et al., 2001
		Summit	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Ursula Rivers	Boskovic and Tobutt, 2001
Group II	*S1S3*	Belle Agathe	Boskovic and Tobutt, 2001
		Bigarreau de Schrecken	Boskovic and Tobutt, 2001
		Black Elton	Boskovic and Tobutt, 2001
		Caroon B	Boskovic and Tobutt, 2001
		Cristalina	Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Erika	Schmidt and Shulze, 1998
		Gil Peck	Tao et al., 1999b
		Koukanishiki	Yamane et al., 2000b
		Merton Crane	Boskovic and Tobutt, 1996
		Oktavia	Schmidt and Shulze, 1998
		Olympus	Sonneveld et al., 2001
		Regina	Schmidt et al., 1999; Choi et al., 2000
		Samba	Wiersma et al., 2001
		Sonnet	Wiersma et al., 2001
		Valeska	Schmidt et al., 1999
		Van	Boskovic and Tobutt, 1996; Tao et al., 1999b; Choi et al., 2000 Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Venus	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Victoria Black A	Boskovic and Tobutt, 2001
		Waterloo	Boskovic and Tobutt, 2001
		Windsor A	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
Group III	*S3S4*	Angela	Wiersma et al., 2001
		Bigarreau Esperen	Boskovic and Tobutt, 2001
		Bing	Boskovic and Tobutt, 1996; Tao et al., 1999b; Choi et al., 2000 Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Buttners	Choi et al., 2000; Boskovic and Tobutt, 2001
		Emperor Francis	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Heinriches Riesen	Boskovic and Tobutt, 2001
		Kristin	Choi et al., 2000; Wiersma et al., 2001
		Lambert	Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Namare	Schmidt et al., 1999
		Namosa	Schmidt et al., 1999
		Napoleon	Boskovic and Tobutt, 1996; Tao et al., 1999b; Choi et al., 2000 Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Querfurter Konigskirsch	Boskovic and Tobutt, 2001
		Somerset	Choi et al., 2000
		Star	Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Turkey Heart B	Choi et al., 2000
		Ulster	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Vernon	Boskovic and Tobutt, 2001

		Yellow Spanish	Boskovic and Tobutt, 2001
Group IV	*S2S3*	Allman Gulrod	Boskovic and Tobutt, 2001
		Cavalier	Hauck et al., 2001
		Kassins	Schmidt et al., 1999
		Kentish Bigarreau	Boskovic and Tobutt, 2001
		Knight's Bigarreau	Boskovic and Tobutt, 2001; Choi et al., 2000
		Late Amber	Boskovic and Tobutt, 2001
		Ludwig's Bigarreau	Boskovic and Tobutt, 2001
		Merton Premier	Boskovic and Tobutt, 2001
		Sue	Tao et al., 1999b; Wiersma et al., 2001
		Vega	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Velvet	Tao et al., 1999b; Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Victor	Tao et al., 1999b; Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 1996, 2001
		Viga	Choi et al., 2000; Wiersma et al., 2001
		Vogue	Choi et al., 2000; Wiersma et al., 2001
Group V	*S4S5*	Turkey Heart	Boskovic and Tobutt, 2001
		Late Bleck Bigarreau	Boskovic and Tobutt, 1996, 2001
Group VI	*S3S6*	Early Amber	Wiersma et al., 2001
		Elton Heart	Boskovic and Tobutt, 2001
		Governor Wood	Boskovic and Tobutt, 1996; Tao et al., 1999b; Choi et al., 2000 Boskovic and Tobutt, 2001
		Heartland	Choi et al., 2000
		Merton Heart	Boskovic and Tobutt, 1996; Boskovic et al., 1997; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Merton Marvel	Boskovic and Tobutt, 2001
		Nanyo	Tao et al.,1999b
		Satonishiki	Tao et al.,1999b
		Gold	Choi et al., 2000; Wiersma et al., 2001; Hauck et al., 2001
		Turkish Black	Boskovic and Tobutt, 2001
Group VII	*S3S5*	Black Eagle A	Boskovic and Tobutt, 2001
		Bradbourne Black	Boskovic and Tobutt, 1996, 2001
		Fruhe Luxburger	Boskovic and Tobutt, 2001
		Goucher	Boskovic and Tobutt, 2001; Hauck et al., 2001
		Hedelfingen	Boskovic and Tobutt, 1996; Schmidt et al., 1999; Wiersma et al., 2001; Choi et al., 2000; Boskovic and Tobutt, 2001 Hauck et al., 2001
		Hooker's Black	Boskovic and Tobutt, 2001
		Nadino	Choi et al., 2000; Hauck et al., 2001
Group VIII	*S2S5*	Malling Black Eagle	Boskovic and Tobutt, 2001
		Vista	Wiersma et al., 2001
Group IX	*S1S4*	Bada	Wiersma et al., 2001
		Black Giant	Wiersma et al., 2001
		Black Republican	Wiersma et al., 2001
		Chinook	Wiersma et al., 2001
		Dawson	Wiersma et al., 2001
		Early Lyons	Wiersma et al., 2001
		Hudson	Choi et al., 2000
		Merton Late	Boskovic and Tobutt, 2001
		Merton Reward	Boskovic and Tobutt, 2001
		Rainier	Tao et al., 1999b; Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001
		Republican	Choi et al., 2000; Wiersma et al., 2001

		Rube	Schmidt et al., 1999
		Salmo	Wiersma et al., 2001; Sonneveld et al., 2001
		Summer Jewel	Wiersma et al., 2001
		Sylvia	Choi et al., 2000; Wiersma et al., 2001; Sonneveld et al., 2001
Group X	*S6S9*	Bigarreau de Jaboulay	Boskovic and Tobutt, 2001
		Bigarreau de Mezel	Boskovic and Tobutt, 2001
		Black Tartarian E	Boskovic and Tobutt, 2001
		Lyons	Wiersma et al., 2001
Group XI	*S2S7*	Cryall's seedling	Choi et al., 2000; Boskovic and Tobutt, 2001
		Early Purple (Hinode)	Yamane et al., 2000a; Hauck et al., 2001
		Guigne d' Annonay	Choi et al., 2000; Boskovic and Tobutt, 2001 Hauck et al., 2001
Group XII	*S6S13*	Noble	Boskovic and Tobutt, 2001; Hauck et al., 2001
Group XIII	*S2S4*	Deacon	Wiersma et al., 2001
		Namada	Schmidt et al., 1999
		Noir de Schemidt	Wiersma et al., 2001
		Ord	Boskovic and Tobutt, 2001
		Patricia	Wiersma et al., 2001
		Peggy Rivers	Boskovic and Tobutt, 2001
		Royalton	Choi et al., 2000
		Sam	Choi et al., 2000; Wiersma et al., 2001
		Schmidt	Choi et al., 2000; Wiersma et al., 2001 Boskovic and Tobutt, 2001
		Vic	Choi et al., 2000; Wiersma et al., 2001 Boskovic and Tobutt, 2001
Group XIV	*S1S5*	Alma	Schmidt and Schulze, 1998
		Allers Spate	Schmidt and Schulze, 1998
		Annabella	Schmidt and Schulze, 1998
		Bianca	Schmidt and Schulze, 1998
		Noir de Guben	Choi et al., 2000; Wiersma et al., 2001 Boskovic and Tobutt, 2001
		Seneca	Choi et al., 2000; Hauck et al., 2001
		Valera	Choi et al., 2000; Wiersma et al., 2001 Boskovic and Tobutt, 2001; Hauck et al., 2001
Group XV	*S5S6*	Colney	Boskovic et al., 1997; Schmidt et al., 1999; Boskovic and Tobutt, 2001
		Erianne	Boskovic and Tobutt, 2001
		Zweitfruhe	Boskovic and Tobutt, 2001
Group XVI	*S3S9*	Burlat	Tao et al., 1999b; Choi et al., 2000; Wiersma et al., 2001; Schmidt et al., 1999; Boskovic and Tobutt, 2001 Hauck et al., 2001
		Moreau	Tao et al., 1999b; Choi et al., 2000; Boskovic and Tobutt, 2001
		Chelan	Choi et al., 2000; Hauck et al., 2001
		Mona	Hauck et al., 2001
		Nabigos	Schmidt et al., 1999
		Naline	Schmidt et al., 1999
		Naprumi	Schmidt et al., 1999
		Tieton	Hauck et al., 2001
Group XVII	*S4S6*	Benishuho	Yamane et al., 2000b
		Elton Heart	Wiersma et al., 2001
		Merton Glory	Yamane et al., 2000b; Boskovic and Tobutt, 2001
		Nutberry Black	Boskovic and Tobutt, 2001

Group XVIII	*S1S9*	Norbury's Early Black	Boskovic and Tobutt, 2001
		Smoky Dun	Boskovic and Tobutt, 2001
Group XIX	*S3S13*	Reverchon	Boskovic and Tobutt, 2001
		Wellington A	Boskovic and Tobutt, 2001
Group XX	*S1S6*	Benisayaka	Yamane et al., 2000b
		Bowyer Heart	Boskovic and Tobutt, 2001
		Mermat	Schmidt et al., 1999
		Rockport Bigarreau (Takasago)	Yamane et al., 2000a
Group XXI	*S4S9*	Inge	Boskovic et al., 1997; Boskovic and Tobutt, 2001 Hauck et al., 2001
		Merpet	Wiersma et al., 2001
Group XXII	*S3S12*	Princess	Wiersma et al., 2001
		Schneiders	Choi et al., 2000; Wiersma et al., 2001; Boskovic and Tobutt, 2001; Hauck et al., 2001
Group O		Universal Donors (compatible with cultivars in Groups I-XIX)	
	S1S7	Charger	Boskovic et al., 1997; Boskovic and Tobutt, 2001; Hauck et al., 2001
	S5S14	Dikkeloen	Boskovic and Tobutt, 2001
	S6S12	Flamentiner	Boskovic and Tobutt, 2001
	S5S13	Goodnestone Black	Boskovic and Tobutt, 2001
	S2S6	Knauffs Riesen	Boskovic and Tobutt, 2001
	S10S11	Orleans 171	Boskovic et al., 1997; Boskovic and Tobutt, 2001; Hauck et al., 2001
	S3Sx	Rodmersham seedling	Boskovic and Tobutt, 2001
	S3Sx	Strawberry Heart	Boskovic and Tobutt, 2001

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